



D4

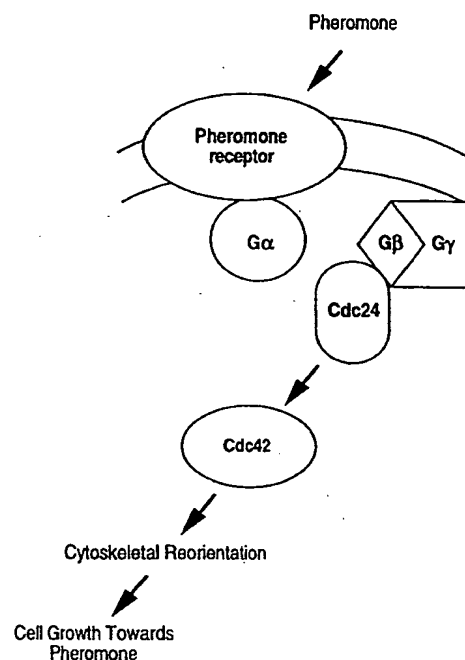
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/31, C07K 14/82, A61K 38/16</b>		<b>A1</b>	(11) International Publication Number: <b>WO 99/18213</b>
			(43) International Publication Date: 15 April 1999 (15.04.99)
(21) International Application Number: PCT/GB98/03033		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 8 October 1998 (08.10.98)			
(30) Priority Data: 9721357.3      8 October 1997 (08.10.97)      GB 9721358.1      8 October 1997 (08.10.97)      GB 9812793.9      12 June 1998 (12.06.98)      GB			
(71) Applicant (for all designated States except US): MEDICAL RESEARCH COUNCIL [GB/GB]; 20 Park Crescent, London W1N 4AL (GB).			
(72) Inventors; and (75) Inventors/Applicants (for US only): ARKOWITZ, Robert, Alan [US/GB]; Division of Cell Biology, MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH (GB). NERN, Peter, Michael, Aljoscha [DE/GB]; Division of Cell Biology, MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH (GB).			
(74) Agents: HARDING, Charles, Thomas et al.; D. Young & Co., 21 New Fetter Lane, London EC4A 1DA (GB).			
		<b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	

(54) Title: MUTANTS OF YEAST Cdc24p, DEFECTIVE IN BINDING OF THE G-PROTEIN BETA SUBUNIT

## (57) Abstract

The pheromone signal transduction in yeasts involves hormone binding to a G-protein coupled membrane receptor, interaction of Cdc24p with G $\beta$ , and ultimately results in polarized growth towards the hormone source (mating partner), caused by changes in the cytoskeleton. The present invention describes three recessive mutants of Cdc24p, at the amino acids 189 and 190, which do not interact with G $\beta$ , and which cause the cytoskeleton to focus adjacent to the last budding site, rather than towards the hormone gradient. In contrast to previously described Cdc24p mutants, those presented here are not affected in their normal vegetative growth and hormone-induced processes, other than cytoskeleton orientation.



**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

## MUTANTS OF YEAST Cdc24p, DEFECTIVE IN BINDING OF THE G-PROTEIN BETA SUBUNIT

The present invention relates to nucleotide sequences and protein sequences. In particular, the present invention relates to nucleotide sequences and protein sequences that affect interactions  
5 of cellular components.

According to Cerione and Zheng (The Dbl family of oncogenes *Current Opinion In Cell Biology* 8, 216-222 (1996)), genetic screening and biochemical studies during the past years have led to the discovery of a certain family of cell growth regulatory proteins and oncogene  
10 products for which the Dbl oncoprotein is the prototype. Another review on Dbl is presented by Machesky and Hall (1996 Trends In Cell Biology 6 pp 3-4-310).

Cerione and Zheng (*ibid*) say that proto-Dbl is a 115 kDa cytoskeleton-associated protein that is found in tissues such as brain, ovary, testis and adrenal glands. Oncogenic activation of proto-  
15 Dbl occurs as a result of an amino-terminal truncation of proto-Dbl which leaves residues 498-925 fused with the product of an as yet unidentified gene which is localised on chromosome 3.

Cerione and Zheng also say that a region located between residues 498 and 674 of proto-Dbl - which is retained by oncogenic Dbl - has significant similarities with the *Saccharomyces*  
20 *cerevisiae* cell division cycle molecule Cdc24p and the breakpoint cluster gene product Bcr (see also Hart *et al* 1991 Nature 354 311-314; Miyamoto *et al* 1991 Biochem Biophys Res Commun 181 604-610; Ron *et al* 1991 New Biol 3 372-379). This region - which is referred to as being the DH domain - was later shown to be responsible for the GEF (GDP-GTP Exchange Factor - otherwise known as a guanine nucleotide exchange factor) activity of the Dbl oncoprotein and to  
25 be critical for its transforming function (see also Hart *et al* J Biol Chem 269 62-65).

Cerione and Zheng also report that since the initial identification of Dbl as a GEF for Rho-type GTP binding proteins, a number of oncogene products and growth regulatory molecules have been shown to contain a DH domain in tandem with another region designated PH (i.e. a  
30 pleckstrin homology domain which is found between residues 703-812 in of proto-Dbl). Many of these products and molecules, such as Bcr, Cdc24, Sos, Vav, ect-2, Ost, Tim, Lbc, Lfc and Dbc, form a family of GEFs which have been implicated in cell growth regulation. Cerione and

Zheng provide details on each of these products and molecules. In addition, these and other products and molecules are discussed below.

Cerione and Zheng (*ibid*) end their Abstract by saying:

“Despite the increasing interest in the Dbl family of proteins, there is still a good deal to learn regarding the biochemical mechanisms that underlie their diverse biological functions.”

As mentioned above, it is known that proto-Dbl has significant similarities with the *S. cerevisiae* cell division cycle molecule Cdc24p which is a GEF for the Rho-family GTPase molecule Cdc42p (see again Hart *et al* 1991 Nature 354 311-314; Miyamoto *et al* 1991 Biochem Biophys Res Commun 181 604-610; Ron *et al* 1991 New Biol 3 372-379; Zheng *et al* 1994 J Biol Chem 269 2369-2372). However, whilst it is known that the Rho-family GTPases and their regulators are essential for cytoskeletal reorganisation and transcriptional activation in response to extracellular signals<sup>1,2</sup>, little is known about what links these molecules to membrane receptors. For example, in the budding yeast *S. cerevisiae*, haploid cells respond to mating pheromone through a G-protein coupled receptor (Ste2p/Ste3p) via Gβγ (Ste4p/Ste18p) resulting in cell cycle arrest, transcriptional activation, and polarised growth towards a mating partner<sup>4,5</sup>. Recently, the Rho-family GTPase Cdc42p and its exchange factor Cdc24p have been implicated in the mating process<sup>6,7</sup> but their specific role is unknown.

However, in our studies (which are presented below) on *S. cerevisiae* we have been able to identify hitherto unrecognised regions that play a key role in the interaction of cellular components. This finding has broad implications - not only for the design of anti-fungal drugs (such as those that could be directed against the yeast *Candida*) but also in the screening and design of agents that can affect oncogenes such as Dbl, in particular proto-Dbl.

Moreover, in our studies (which are presented below), we have identified novel *cdc24* alleles which do not affect vegetative growth but drastically reduce the ability of yeast cells to mate. When exposed to mating pheromone these mutants arrest growth, activate transcription, and undergo characteristic morphological and actin cytoskeleton polarisation. However, the mutants

are unable to orient towards a pheromone gradient and instead position their mating projection adjacent to their previous bud site. Strikingly, these mutants are specifically defective in the binding of Cdc24p to G $\beta\gamma$ . This work demonstrates that the association of a GEF and the  $\beta\gamma$ -subunit of a hetero-trimeric G-protein (G $\beta\gamma$ ) links receptor-mediated activation to oriented cell growth.

The present invention also demonstrates that Far1, a cyclic dependent kinase inhibitor (CDK1) may also be implicated as being important for orientated cell growth.

Thus, according to one broad aspect of the present invention there is provided a GEF capable of interacting with a G $\beta$  such that the interaction provides a connection between G protein coupled receptor activation and polarised cell growth.

According to another broad aspect of the present invention there is also provided an agent capable of affecting a GEF/G $\beta$  interaction, which interaction provides a connection between G protein coupled receptor activation and polarised cell growth.

These and other aspects of the present invention are set out in the claims.

By way of example, in a broad aspect, the present invention provides a nucleotide sequence shown as SEQ I.D. No. 1 or a derivative, fragment, variant or homologue thereof, wherein the expression product of the nucleotide sequence has the capability of not substantially affecting the interaction of G $\beta$  with GEF or a homologue thereof that is usually capable of being associated therewith.

The term "expression product of the nucleotide sequence has the capability of not substantially affecting the interaction of G $\beta$  with GEF or a homologue thereof that is usually capable of being associated therewith" means that if the expression product were to be present within GEF and the GEF were to be contacted with G $\beta$  then the expression product would not substantially affect the interaction of G $\beta$  with GEF.

Thus, alternatively expressed, the present invention covers a nucleotide sequence shown as SEQ I.D. No. 1 or a derivative, fragment, variant or homologue thereof, wherein the expression product of the nucleotide sequence has the capability of not substantially affecting the interaction of G $\beta$  with GEF or a homologue thereof that is usually capable of being associated therewith if the expression product were to be present within GEF and the GEF were to be contacted with G $\beta$ .

With this aspect of the present invention, the expression product need not necessarily be present within GEF and/or the GEF need not necessarily be contacted with G $\beta$ . By way of example, the expression product can be part of a truncated GEF and/or part of a fused protein. However, if the expression product were present within GEF, then preferably the GEF is not in its natural environment. By way of example, the GEF can be in an isolated form - such as in an assay device. Likewise, if the expression product were contacted with G $\beta$  then preferably the G $\beta$  is not in its natural environment. By way of example, the G $\beta$  can be in an isolated form - such as in an assay device.

The present invention also covers a mutant of the nucleotide sequence shown as SEQ I.D. No. 1 or a derivative, fragment, variant or homologue thereof, wherein the expression product of the mutant nucleotide sequence has the capability of substantially affecting the interaction of G $\beta$  with GEF or a homologue thereof that is usually capable of being associated therewith.

The term "expression product of the mutant nucleotide sequence has the capability of substantially affecting the interaction of G $\beta$  with GEF or a homologue thereof that is usually capable of being associated therewith" means that if the expression product were to be present within a GEF like entity (such as GEF bearing that mutation) and that GEF like entity were to be contacted with G $\beta$  then the expression product would substantially affect the interaction of G $\beta$  with that GEF like entity.

Thus, alternatively expressed, the present invention also covers a mutant of the nucleotide sequence shown as SEQ I.D. No. 1 or a derivative, fragment, variant or homologue thereof, wherein the expression product of the mutant nucleotide sequence has the capability of substantially affecting the interaction of G $\beta$  with GEF or a homologue thereof that is usually

capable of being associated therewith if the expression product were to be present within GEF and the GEF were to be contacted with G $\beta$ .

With this aspect of the present invention, the expression product need not necessarily be present within the GEF like entity and/or the GEF like entity need not necessarily be contacted with G $\beta$ . By way of example, the expression product can be part of a truncated GEF and/or part of a fused protein. The GEF like entity may be in an isolated form - such as in an assay device. Likewise, if the expression product were contacted with G $\beta$  then preferably the G $\beta$  is not in its natural environment. By way of example, the G $\beta$  can be in an isolated form - such as in an assay device.

In one preferred aspect, the GEF is Cdc24p. Other suitable GEFs have been mentioned above.

Thus, the present invention also covers in a broad aspect a nucleotide sequence shown as SEQ I.D. No. 1 or a derivative, fragment, variant or homologue thereof, wherein the expression product of the nucleotide sequence has the capability of not substantially affecting the interaction of G $\beta$  with Cdc24p or a homologue thereof that is usually capable of being associated therewith.

The term "expression product of the nucleotide sequence has the capability of not substantially affecting the interaction of G $\beta$  with Cdc24p or a homologue thereof that is usually capable of being associated therewith" means that if the expression product were to be present within Cdc24p and the Cdc24p were to be contacted with G $\beta$  then the expression product would not substantially affecting the interaction of G $\beta$  with Cdc24p.

Thus, alternatively expressed, the present invention covers in a broad aspect a nucleotide sequence shown as SEQ I.D. No. 1 or a derivative, fragment, variant or homologue thereof, wherein the expression product of the nucleotide sequence has the capability of not substantially affecting the interaction of G $\beta$  with Cdc24p or a homologue thereof that is usually capable of being associated therewith if the expression product were to be present within Cdc24p and the Cdc24p were to be contacted with G $\beta$ .

With this aspect of the present invention, the expression product need not necessarily be present within Cdc24p and/or the Cdc24p need not necessarily be contacted with G $\beta$ . By way of example, the expression product can be part of a truncated Cdc24p and/or part of a fused protein. However, if the expression product is present within Cdc24p, then preferably the Cdc24p is not in its natural environment. By way of example, the Cdc24p can be in an isolated form - such as in an assay device. Likewise, if the expression product were contacted with G $\beta$  then preferably the G $\beta$  is not in its natural environment. By way of example, the G $\beta$  can be in an isolated form - such as in an assay device.

By way of further example, the present invention also covers a mutant of the nucleotide sequence shown as SEQ I.D. No. 1 or a derivative, fragment, variant or homologue thereof, wherein the expression product of the mutant nucleotide sequence has the capability of substantially affecting the interaction of G $\beta$  with Cdc24p or a homologue thereof that is usually capable of being associated therewith.

The term "expression product of the mutant nucleotide sequence has the capability of substantially affecting the interaction of G $\beta$  with Cdc24p or a homologue thereof that is usually capable of being associated therewith" means that if the expression product were to be present within a Cdc24p like entity (such as Cdc24p bearing that mutation) and that Cdc24p like entity were to be contacted with G $\beta$  then the expression product would substantially affect the interaction of G $\beta$  with that Cdc24p like entity.

With this aspect of the present invention, the expression product need not necessarily be present within the Cdc24p like entity and/or the Cdc24p like entity need not necessarily be contacted with G $\beta$ . By way of example, the expression product can be part of a truncated Cdc24p and/or part of a fused protein. The Cdc24p like entity may be in an isolated form - such as in an assay device. Likewise, if the expression product were contacted with G $\beta$  then preferably the G $\beta$  is not in its natural environment. By way of example, the G $\beta$  can be in an isolated form - such as in an assay device.

In a preferred aspect, the present invention covers the sequences of the present invention in isolated form - in other words the sequences are not in their natural environment and when



they have been expressed by their natural coding sequences which are under the control of their natural expression regulatory elements (such as the natural promoter etc.). By way of example the sequences may be in an assay device.

5 It is to be noted that the nucleotide sequence presented as SEQ ID No. 1 is quite different to the DH domain and the PH domain discussed by Cerione and Zheng (*ibid*). It is also to be noted that the nucleotide sequence presented as SEQ ID No. 1 is in a region quite different to the DH domain and the PH domain.

10 One important aspect of the present invention is that we have found it is possible to affect the interaction of Cdc24p with a  $\beta$  subunit (such as Ste4p) or even a  $\beta\gamma$  subunit (such as Ste4p/Ste18p) of a hetero-trimeric G-protein (hereinafter collectively referred to as "G $\beta$ "). If the interaction is detrimentally affected (such as lost) then this may in turn prevent (or at least reduce) signalling (possibly GEF activity) being passed to the the Rho-family GTPase (Cdc42p). Hence, the present invention also covers the use of any one or more of the  
15 the Rho-family GTPases.

The term "derivative, fragment, variant or homologue" in relation to the nucleotide Sequence ID No. 1 of the present invention includes any substitution of, modification of, replacement of,  
20 deletion of or addition of one (or more) nucleic acid from or to the sequence providing the resultant nucleotide sequence or the expression product thereof has the capability of not substantially affecting the interaction of G $\beta$  with Cdc24p or a homologue thereof that is usually capable of being associated with the Cdc24p or the homologue thereof. In particular, the term  
25 "homologue" covers homology with respect to function. With respect to sequence homology (i.e. similarity), preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to the sequence shown as SEQ ID No.1 in the attached sequence listings. More preferably there is at least 95%, such as at least 98%, homology to the sequence shown as SEQ ID No. 1 in the attached sequence listings.

30 The term "derivative, fragment, variant or homologue" in relation to the protein Sequence ID No. 2 of the present invention includes any substitution of, modification of, replacement of,

deletion of or addition of one (or more) amino acid from or to the sequence providing the resultant amino acid sequence has the capability of not substantially affecting the interaction of G $\beta$  with Cdc24p or a homologue thereof that is usually capable of being associated with the Cdc24p or the homologue thereof. In particular, the term "homologue" covers homology with respect to function. With respect to sequence homology (i.e. similarity), preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to the sequence shown as SEQ ID No.2 in the attached sequence listings. More preferably there is at least 95%, such as at least 98%, homology to the sequence shown as SEQ ID No. 2 in the attached sequence listings.

An example of a fragment of the expression product of SEQ ID No. 1 that has the capability of not substantially affecting the interaction of G $\beta$  with Cdc24p or a homologue thereof that is usually capable of being associated with the Cdc24p or the homologue thereof is the amino acid sequence presented as SEQ ID No. 15 or SEQ ID No. 16. The present invention also covers nucleotide sequences coding for such sequences.

With respect to the mutated sequences then, in a preferred aspect, the mutated sequence comprises one or more mutations in the region presented as SEQ ID No. 15 or SEQ ID No. 16.

An example of a fragment of the expression product of a mutant SEQ ID No. 1 that has the capability of substantially affecting the interaction of G $\beta$  with Cdc24p or a homologue thereof that is usually capable of being associated with the Cdc24p or the homologue thereof is the amino acid sequence presented as SEQ ID No. 17 or SEQ ID No. 18 or SEQ ID No. 19. The present invention also covers nucleotide sequences coding for such sequences.

In particular, the term "homology" as used herein may be equated with the term "identity". Relative sequence homology (i.e. sequence identity) can be determined by commercially available computer programs that can calculate % homology between two or more sequences. Typical examples of such computer programs are BLAST and CLUSTAL.

Sequence homology (or identity) may moreover be determined using any suitable homology algorithm, using for example default parameters. Advantageously, the BLAST algorithm is

employed, with parameters set to default values. The BLAST algorithm is described in detail at [http://www.ncbi.nih.gov/BLAST/blast\\_help.html](http://www.ncbi.nih.gov/BLAST/blast_help.html), which is incorporated herein by reference. The search parameters are defined as follows, and are advantageously set to the defined default parameters.

Advantageously, "substantial homology" when assessed by BLAST equates to sequences which match with an EXPECT value of at least about 7, preferably at least about 9 and most preferably 10 or more. The default threshold for EXPECT in BLAST searching is usually 10.

BLAST (Basic Local Alignment Search Tool) is the heuristic search algorithm employed by the programs *blastp*, *blastn*, *blastx*, *tblastn*, and *tblastx*; these programs ascribe significance to their findings using the statistical methods of Karlin and Altschul (see [http://www.ncbi.nih.gov/BLAST/blast\\_help.html](http://www.ncbi.nih.gov/BLAST/blast_help.html)) with a few enhancements. The BLAST programs were tailored for sequence similarity searching, for example to identify homologues to a query sequence. The programs are not generally useful for motif-style searching. For a discussion of basic issues in similarity searching of sequence databases, see Altschul *et al* (1994) *Nature Genetics* 6:119-129.

The five BLAST programs available at <http://www.ncbi.nlm.nih.gov> perform the following tasks:

**blastp** compares an amino acid query sequence against a protein sequence database;

**blastn** compares a nucleotide query sequence against a nucleotide sequence database;

**blastx** compares the six-frame conceptual translation products of a nucleotide query sequence (both strands) against a protein sequence database;

**tblastn** compares a protein query sequence against a nucleotide sequence database dynamically translated in all six reading frames (both strands).

**tblastx** compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database.

BLAST uses the following search parameters:

5

**HISTOGRAM** Display a histogram of scores for each search; default is yes. (See parameter H in the BLAST Manual).

10

**DESCRIPTION** nucleotide sequence Restricts the number of short descriptions of matching sequences reported to the number specified; default limit is 100 descriptions. (See parameter V in the manual page). See also **EXPECT** and **CUTOFF**.

15

**ALIGNMENTS** Restricts database sequences to the number specified for which high-scoring segment pairs (HSPs) are reported; the default limit is 50. If more database sequences than this happen to satisfy the statistical significance threshold for reporting (see **EXPECT** and **CUTOFF** below), only the matches ascribed the greatest statistical significance are reported. (See parameter B in the BLAST Manual).

20

**EXPECT** The statistical significance threshold for reporting matches against database sequences; the default value is 10, such that 10 matches are expected to be found merely by chance, according to the stochastic model of Karlin and Altschul (1990). If the statistical significance ascribed to a match is greater than the **EXPECT** threshold, the match will not be reported. Lower **EXPECT** thresholds are more stringent, leading to fewer chance matches being reported. Fractional values are acceptable. (See parameter E in the BLAST Manual).

25

**CUTOFF** Cutoff score for reporting high-scoring segment pairs. The default value is calculated from the **EXPECT** value (see above). HSPs are reported for a database sequence only if the statistical significance ascribed to them is at least as high as would be ascribed to a lone HSP having a score equal to the **CUTOFF** value. Higher **CUTOFF** values are more stringent, leading to fewer chance matches being reported. (See parameter S in the BLAST

30

Manual). Typically, significance thresholds can be more intuitively managed using EXPECT.

MATRIX Specify an alternate scoring matrix for BLASTP, BLASTX, TBLASTN and  
5 TBLASTX. The default matrix is BLOSUM62 (Henikoff & Henikoff, 1992). The valid alternative choices include: PAM40, PAM120, PAM250 and IDENTITY. No alternate scoring matrices are available for BLASTN; specifying the MATRIX directive in BLASTN requests returns an error response.

10 STRAND Restrict a TBLASTN search to just the top or bottom strand of the database sequences; or restrict a BLASTN, BLASTX or TBLASTX search to just reading frames on the top or bottom strand of the query sequence.

FILTER Mask off segments of the query sequence that have low compositional  
15 complexity, as determined by the SEG program of Wootton & Federhen (1993) Computers and Chemistry 17:149-163, or segments consisting of short-periodicity internal repeats, as determined by the XNU program of Claverie & States (1993) Computers and Chemistry 17:191-201, or, for BLASTN, by the DUST program of Tatusov and Lipman (see <http://www.ncbi.nlm.nih.gov>). Filtering can eliminate statistically significant but  
20 biologically uninteresting reports from the blast output (e.g., hits against common acidic-, basic- or proline-rich regions), leaving the more biologically interesting regions of the query sequence available for specific matching against database sequences.

Low complexity sequence found by a filter program is substituted using the letter "N" in  
25 nucleotide sequence (e.g., "NNNNNNNNNNNNNN") and the letter "X" in protein sequences (e.g., "XXXXXXXXXX").

Filtering is only applied to the query sequence (or its translation products), not to database sequences. Default filtering is DUST for BLASTN, SEG for other programs.

30

It is not unusual for nothing at all to be masked by SEG, XNU, or both, when applied to sequences in SWISS-PROT, so filtering should not be expected to always yield an effect.

Furthermore, in some cases, sequences are masked in their entirety, indicating that the statistical significance of any matches reported against the unfiltered query sequence should be suspect.

- 5 NCBI-gi Causes NCBI gi identifiers to be shown in the output, in addition to the accession and/or locus name.

Most preferably, sequence comparisons are conducted using the simple BLAST search algorithm provided at <http://www.ncbi.nlm.nih.gov/BLAST>.

10

Other computer program methods to determine identify and similarity between the two sequences include but are not limited to the GCG program package (Devereux *et al* 1984 Nucleic Acids Research 12: 387 and FASTA (Atschul *et al* 1990 J Molec Biol 403-410).

- 15 The term "variant" also encompasses sequences that are complementary to sequences that are capable of hybridising to the nucleotide sequences presented herein.

Preferably, the term "variant" encompasses sequences that are complementary to sequences that are capable of hybridising under stringent conditions (eg. 65°C and 0.1xSSC {1xSSC =  
20 0.15 M NaCl, 0.015 M Na<sub>3</sub> citrate pH 7.0}) to the nucleotide sequences presented herein.

The present invention also relates to nucleotide sequences that can hybridise to the nucleotide sequences of the present invention (including complementary sequences of those presented herein).

25

The present invention also relates to nucleotide sequences that are complementary to sequences that can hybridise to the nucleotide sequences of the present invention (including complementary sequences of those presented herein).

- 30 The term "hybridization" as used herein shall include "the process by which a strand of nucleic acid joins with a complementary strand through base pairing" (Coombs J (1994) Dictionary of Biotechnology, Stockton Press, New York NY) as well as the process of

amplification as carried out in polymerase chain reaction technologies as described in Dieffenbach CW and GS Dveksler (1995, PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview NY).

5 Also included within the scope of the present invention are polynucleotide sequences that are capable of hybridizing to the nucleotide sequence of the present invention or other nucleotide sequences coding for the protein sequence of the present invention under conditions of intermediate to maximal stringency. Hybridization conditions are based on the melting temperature ( $T_m$ ) of the nucleic acid binding complex, as taught in Berger and  
10 Kimmel (1987, Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol 152, Academic Press, San Diego CA), and confer a defined "stringency" as explained below.

Maximum stringency typically occurs at about  $T_m - 5^\circ\text{C}$  ( $5^\circ\text{C}$  below the  $T_m$  of the probe);  
15 high stringency at about  $5^\circ\text{C}$  to  $10^\circ\text{C}$  below  $T_m$ ; intermediate stringency at about  $10^\circ\text{C}$  to  $20^\circ\text{C}$  below  $T_m$ ; and low stringency at about  $20^\circ\text{C}$  to  $25^\circ\text{C}$  below  $T_m$ . As will be understood by those of skill in the art, a maximum stringency hybridization can be used to identify or detect identical polynucleotide sequences while an intermediate (or low) stringency hybridization can be used to identify or detect similar or related polynucleotide  
20 sequences.

In a preferred aspect, the present invention covers nucleotide sequences that can hybridise to the nucleotide sequence of the present invention under stringent conditions (e.g.  $65^\circ\text{C}$  and  $0.1\times\text{SSC}$ ).

25 Examples of homologues of Cdc24p include but are not limited to any one or more of the homologues listed above or below, such as proto-Dbl, Bcr, Sos, Vav, ect-2, Ost, Tim, Lbc, Lfc and Dbc.

30 The term "mutant" in relation to the nucleotide sequence of the present invention means a variant of SEQ ID No. 1 but wherein that variant or the expression product thereof has the

capability of substantially affecting the interaction of G $\beta$  with Cdc24p or a homologue thereof that is usually capable of being associated with the Cdc24p or the homologue thereof.

Preferred mutants of the nucleotide sequence of the present invention include any one or more of the nucleotide sequences presented as SEQ ID No. 3, SEQ ID No. 5 or SEQ ID No. 7.

The term "mutant" in relation to the protein sequence of the present invention means a variant of SEQ ID No. 2 but wherein that variant has the capability of substantially affecting the interaction of G $\beta$  with Cdc24p or a homologue thereof that is usually capable of being associated with the Cdc24p or the homologue thereof.

Preferred mutants of the protein sequence of the present invention include any one or more of the protein sequences presented as SEQ ID No. 4, SEQ ID No. 6 or SEQ ID No. 8.

The term "growth behaviour" includes growth *per se* (but not vegetative growth of yeast), growth control and growth orientation of cells. In some aspects, it includes at least growth orientation of cells. The term may also include the mating pattern (e.g. mating *per se* or mating behaviour) of cells.

For a preferred aspect of the present invention, any one or more of the nucleotide sequence of the present invention or the expression product thereof, or the mutant nucleotide sequence of the present invention or the expression product thereof, or the protein of the present invention, or the mutant protein of the present invention may be within a transgenic organism or cell (such as being an integral part thereof) - that is an organism or cell that is not a naturally occurring organism or cell and wherein the organism or cell has been prepared by use of recombinant DNA techniques. The transgenic cell may be part of or contained within tissue.

Preferably, the transgenic organism or cell is a yeast, an animal (such as a mammal) or an animal cell (such as a mammalian cell).

In preferred embodiments, the transgenic organism is a transgenic yeast or a transgenic mouse.



Transgenic yeast may be prepared by appropriately adapting the teachings of Ito *et al* Journal of Bacteriology 153 163-168; Rose *et al* 1991 *Methods in yeast genetics: a laboratory course manual* Cold Spring Harbor, N.Y.: Cold Spring Harbor Press).

Transgenic mammals or mammalian cells may be prepared by appropriately adapting the teachings of Ausubel *et al* 1992 *Short Protocols in Molecular Biology* 2nd Ed. New York: John Wiley and Sons).

The transgenic organism or transgenic cell of the present invention therefore provides a simple assay system that can be used to determine whether one or more agents (e.g. compounds or compositions) have one or more beneficial properties. By way of example, the assay system of the present invention may utilise a mating phenotype and/or the assay system may be a two-hybrid interaction assay.

By way of example, if the transgenic organism is a transgenic yeast which comprises the nucleotide sequence presented as SEQ ID No. 1 or the expression product thereof (namely the protein sequence presented as SEQ ID No. 2) then the yeast could be used to screen for agents that bind to this nucleotide sequence or the expression product thereof and in doing so affect the growth behaviour of the yeast. If an agent produces such a detrimental effect (such as drastically reducing the ability of the yeast to mate), then that agent may also affect the interaction of G $\beta$  with Cdc24p or another Cdc24p entity that is usually capable of being associated therewith. This aspect of the present invention could allow workers to screen for anti-fungal agents, such as agents that could be used to treat or combat *Candida*.

By way of further example, if the transgenic organism is a transgenic yeast which comprises the nucleotide sequence presented as SEQ ID No. 1 or the expression product thereof then the yeast could be used to screen for agents that bind to this nucleotide sequence or expression product thereof and in doing so affect the growth behaviour of the yeast. If an agent produces a detrimental affect (such as drastically reducing the ability of the yeast to mate), then that agent is likely to detrimentally affect the interaction of G $\beta$  with a homologue of Cdc24p with which it is usually capable of being associated. This could allow workers to screen for

compounds or compositions that could for example influence the *in vivo* expression or behaviour of effect of proto-oncogenes and the like - such as proto-Dbl.

By way of further example, if the transgenic organism is a transgenic yeast which comprises a mutant of the nucleotide sequence in accordance with the present invention then the yeast could be used to screen for agents that affect the growth behaviour of the yeast. If an agent produces a marked affect - such as restoration to a normal growth behaviour or a further detrimental growth behaviour - then workers could screen for compounds or compositions that could for example influence the *in vivo* expression or behaviour or effect or activity of a Cdc24 homologue, such as, but not limited to proto-oncogenes such as Dbl and/or Vav.

By way of further example, if the transgenic organism is a transgenic yeast which comprises a homologue (e.g. Dbl) of the nucleotide sequence shown as SEQ ID No. 1 or an expression product thereof then workers could see if that homologue or the expression product thereof had an effect on the growth behaviour of yeast, and thus also to see if it had an effect on the interaction of G $\beta$  with a homologue of Cdc24p. In addition, workers could use those transgenic yeast to screen for agents that modified the effect - such as enhance the growth behaviour or detrimentally affect the growth behaviour. In this aspect, agents that affect the growth behaviour may also influence the activity of oncogenes (or even parts thereof) and therefore have potential as therapeutic agents.

The assays of the present invention may also be used to screen for agents that affect the interaction of Cdc24p or a Cdc24p homologue with G $\beta$  to determine whether that effect has a downstream effect on a Rho-family GTPase.

25

For example, with the present invention - such as by use of the assays of the present invention - it is possible to devise and/or to screen for peptide inhibitors which block GEF/G $\beta$  interaction. In this regard, peptides and peptidyl derivatives based regions encompassing mutants may be used to block and/or antagonise GEF (such as the proto-oncogenes Dbl or Vav) G $\beta$  interaction. Derivatives of these peptides (including peptide mimics) which bind with higher affinity may also be used. The perturbation of these interactions may be of therapeutic value for example in treatment of cancers.

30

In addition, by use of the present invention it is possible to devise simple yeast based assay systems (utilising mating function and interaction reporters). These assay systems will be extremely useful for high through-put screening to identify molecules perturbing the GEF/G $\beta$  interaction.

In addition, it is possible to devise and/or screen for agents that can modulate (e.g. interact), preferably selectively modulate (interact), with and affect Cdc24p/G $\beta$  interactions. Hence, it would be possible to devise and/or to screen for anti-fungal agents directed at invasive and/or pathogenic yeasts such as, but not limited to *Candida albicans* and/or *Cryptococcus neoformans*.

If the assay of the present invention utilises a transgenic organism according to the present invention then transgenic organism may comprise nucleotide sequences etc. that are additional to the nucleotide sequences of the present invention in order to maintain the viability of the transgenic organism.

In the assays of the present invention, the agent can be any suitable compound, composition as well as being (or even including) a nucleotide sequence of interest or the expression product thereof. Hence, if any one of the nucleotide sequences of the present invention are contained within a transgenic organism - such as a transgenic yeast - then that transgenic organism may also contain that nucleotide sequence of interest. If the agent is a nucleotide sequence, then the agent may be, for example, nucleotide sequences from organisms (e.g. higher organisms - such as eukaryotes) that restore or increase the growth behaviour. Agents which affect the growth behaviour may also influence the activity of homologous oncogenes and may therefore be potential therapeutic agents.

The following samples were deposited in accordance with the Budapest Treaty at the recognised depositary of The National Collections of Industrial and Marine Bacteria Limited (NCIMB) at 23 St. Machar Drive, Aberdeen, Scotland, United Kingdom, AB2 1RY on 3 October 1997:

5 *E.coli* CMK603 PRS414CDC24 (WT) - Deposit Number NCIMB 40898

*E.coli* CMK603 PRS414CDC24 (M1) - Deposit Number NCIMB 40899

*E.coli* CMK603 PRS414CDC24 (M2) - Deposit Number NCIMB 40900

10

*E.coli* CMK603 PRS414CDC24 (M3) - Deposit Number NCIMB 40901

Deposit NCIMB 40898 is in respect of *cdc24* (wt); Deposit NCIMB 40899 is in respect of *cdc24-m1*. Deposit NCIMB 40900 is in respect of *cdc24-m2*; Deposit NCIMB 40901 is in  
15 respect of *cdc24-m3*.

In accordance with a preferred aspect of the present invention, the nucleotide sequence is obtainable from, or the protein is expressable from the nucleotide sequence contained within, the respective deposit. By way of example, the respective nucleotide sequence may be isolated from  
20 the respective deposit by use of appropriate restriction enzymes or by use of PCR techniques.

The present invention will now be described only by way of example, in which reference is made to the following Figures:

25 Figure 1 which presents some photographs and a graph;

Figure 2 which presents some images and graphs;

Figure 3 which presents some photographs, a sequence, and a pictorial representation of Cdc24  
30 and DBD Cdc24; and

Figure 4 which presents a pictorial representation of a cellular interaction.

The Figures are discussed in more detail later on.

## Materials and Methods

5

### General techniques

Strains were constructed using standard techniques<sup>21</sup>. All constructs were verified by DNA dye terminator cycle sequencing (ABI377 sequencer).

10

### Strains

pRS414CDC24 contains the *CDC24* ORF including 258 bp upstream of ATG.

- 15 Oligonucleotide-directed mutagenesis was used to introduce silent base changes that resulted in the following ten new restriction sites in *CDC24*: *NheI* (bp -12), *KasI* (bp 283), *AatII* (bp 681), *PstI* (bp 1207), *RsrII* (bp 1369), *BstEII* (bp 1426), *XhoI* (bp 1758), *MluI* (bp 1963), *SalI* (bp 2061), *BamHI* (bp 2485). RAY410 (*MATa*, *leu2*, *CDC24::LEU2*, *ade2*, *lys2*, *his3*, *trp1*, *ura3*, pEG(KT)CDC24) was derived from the diploid YOC380<sup>22</sup> which was transformed with
- 20 pEG(KT)CDC24<sup>23</sup> and sporulated. RAY950 is isogenic to RAY410 but has pRS416GalHis<sub>6</sub>CDC24 as a rescuing plasmid. RAY928 (*MATa*, *leu2-3*, 112, *ura3-52*, *his3-D200*, *trp1-D901*, *lys2-801*, *suc2-D9*, *CDC24::HIS5* pEG[KT]CDC24) and RAY931 (same as RAY928 but *MATa*, *ade2*, *LYS2*) were made by transformation of SEY6210 and 6211 with pEG(KT)CDC24 followed by PCR-based gene disruption of *CDC24*. The *CDC24* ORF was
- 25 replaced with *S. pombe HIS5*<sup>24</sup>, flanked by *LoxP* sites. Replacement of *CDC24* in SEY6211 with a PCR-generated integration cassette consisting of *TRP1* fused to 343 bp of *CDC24* promoter followed by 1704 bp of *CDC24* or *cdc24-m1* ORF was used to construct RAY1034 or RAY1035, respectively.

# IDENTIFICATION OF *cdc24* MUTANTS WITH SPECIFIC DEFECTS IN CELL MATING:

## 5 A) Construction of a library of *cdc24* random mutants

Error-prone PCR was used to generate a library of *cdc24* mutants in a plasmid vector suitable for phenotypic screening in yeast.

### 10 1) Plasmid:

pRS414 *CDC24* with upstream region and new restriction sites (referred to as pRS414CDC24).

### 2) Mutagenic PCRs:

15 Conditions from Fromant, M., Blanquet, S. & Plateau, P. Direct random mutagenesis of gene-sized DNA fragments using polymerase chain-reaction. *Analytical Biochemistry* 224, 347-353 (1995).

20 Different PCR-conditions were tested and the error-rate was determined by DNA sequencing. The following conditions were used for constructing the library used in the screen.

Composition of PCR-reactions (25 µl each):

25 DNA pRS414CDC24 600pM

dATP	0.23 mM
dCTP	0.20 mM
dTTP	2.9 mM
30 dGTP	0.42 mM

Buffer	PCR Buffer supplied with Taq-polymerase
--------	---

MgCl <sub>2</sub>	4 mM
MnCl <sub>2</sub>	0.5 mM
Taq (Ampli-Taq)	2 U per reaction

5

Primer:	~ 0.5 mM
---------	----------

PCR-cycles:

10	step 1	94 °C	5 min
	step 2	91 °C	1 min
	step 3	51 °C	1 min
	step 4	72 °C	3 min
	step 5	72 °C	5 min
15	step 6	4 °C	pause

16 cycles (steps 2-4)

### 3) Library construction:

20

The PCR products were digested with *Aat*II and *Nhe*I (680 bp corresponding to amino acid 1 - 227) were mutagenised and the resulting fragment ligated into pRS414CDC24 (cut with the same enzymes). Ligations were transformed into *E. coli* by electroporation and > 50,000 transformants pooled for plasmid isolation.

25

### B) Phenotypic screening for cell-mating specific *cdc24* alleles

Rationale:

30 To identify mutant *cdc24* alleles which cause defects in cell mating but allow vegetative growth. Yeast strain RAY950, in which expression of CDC24 is repressed in glucose medium, was used.

1) Library plasmids were transformed into RAY950 and transformants selected on SC -trp plates which contained 2% glucose. As RAY950 does not grow on glucose plates this procedure eliminated all non-functional *cdc24* mutants.

2) Transformants were replica-plated onto a lawn of WT (screen 1) or  $\Delta fus1 \Delta fus2$  (screen 2) tester cells, incubated at 30°C for 3 hrs and replica-plated onto plates selecting for diploids or RAY950 derived haploids. Mating defective mutants were identified by comparing the pattern of colonies on the two sets of plates and candidate mutants were picked from the original transformation plates for retesting.

3) Plasmids from mutants were isolated by transformation into *E. coli*. Isolated plasmids were retransformed into RAY950, RAY928 and RAY931 for independent confirmation of phenotype and retested for defects in cell mating.

4) Mutations of confirmed mutants were identified by DNA sequencing. Multiple mutations were separated by subcloning and the mutation responsible for the phenotype identified by mating tests in RAY950.

5) A total of ~ 5,000 yeast transformants were tested in each screen.

- Screen 1 identified two mutants (*cdc24-m1*, *cdc24-m2*).
- Screen 2 identified one mutant (*cdc24-m3*).

#### Phenotypic analyses

Quantitative matings<sup>10</sup>, matings in the presence of saturating pheromone<sup>13</sup>, halo-assays<sup>26</sup> using *sst1::URA3* strains, and *Fus1lacZ* measurements with pSG231<sup>11</sup> were carried out as described. Halo assays showed *MATa* and *MATa cdc24-m1* cells secreted  $\alpha$ -factor and  $\alpha$ -factor, respectively. Actin was visualised with rhodamine phalloidin<sup>27</sup> on a Biorad-MRC-600 confocal microscope and pictures are projections of 4-6 0.5 mm z-series steps. For  $\alpha$ -factor treatment, cells were incubated with 5 mM  $\alpha$ -factor for 2 hr. RAY1034 and RAY1035 cells were used to determine bud scar positions on zygotes<sup>14</sup> visualised with Calcofluor<sup>28</sup>. Similar results were



observed with the position of the bud scar on shmoos. Direct measurement of cell orientation in a pheromone gradient was carried out essentially as described<sup>12</sup>. A pheromone gradient was generated using a micropipet filled with 80 mM a-factor injected at 105 kPa into 1ml of YEPD media layered on top of cells embedded in 2% Low Melting Point (LMP) agarose. Cells shape was recorded by video microscopy on a heated stage at 35° for 4 - 7 hr and data analysis was from traced cell outlines<sup>14</sup>. Mating projections were formed at the same pheromone concentrations and budding, that is non-responding cells were seen at similar distances from the micropipet in both strains.

## Two-Hybrid methods

*STE4*, *BEM1* (372 - 551 aa), *CDC42*[C178S], and *CDC24* /*cdc24-m1* (1-288, 1-160, and 170-245 aa) were cloned by PCR into pGAD424 (AD, *GAL4* activation domain) or pAS1 (DBD, *GAL4* DNA binding domain). Plasmids were transformed into HF7c. For determination of *STE18* requirement, PCR-based gene disruption was carried out in PJ69-4A (*MATa*, *trp1-901*, *leu2-3,112*, *ura3-52*, *his3-200*, *gal4D*, *gal80D*, *GAL2-ADE2*, *LYS2::GAL1-HIS3*, *met2::GAL7-lacZ*)<sup>29</sup>, replacing the entire *STE18* ORF with *K. Lactis URA3*<sup>30</sup>. For all two-hybrid experiments, equal amounts of transformants were spotted on SC-leu-trp and SC-leu-trp-his plates, identical results were obtained with at least four transformants, and for *Dste18* two independent deletion strains. All strains for two-hybrid analyses expressed similar amounts of AD- and DBD- fusion proteins of the expected sizes, as determined by SDS-PAGE and immuno-blotting. None of the DBD fusions showed any self-activation using two different non-interacting AD fusions.

## *In vitro* binding studies

A fragment of *CDC24* (1-472 aa) in pGEX-2T (Pharmacia) and His<sub>6</sub>Ste4p (pTrcSte4) were expressed in *E. coli*. Cells were resuspended in buffer A (PBS, 0.1% TX-100, Phenyl Methyl Sulfonyl Fluoride (PMSF), leupeptin, chymostatin, pepstatin, aprotinin) and lysed by snap freezing in liquid nitrogen followed by sonication. Insoluble material was removed by centrifugation (10,000g). Mixed supernatants (denoted cell extracts) containing His<sub>6</sub>Ste4 and GSTCdc24 fusions were incubated with GSH-agarose (Sigma Chemical Co.) at 4° for 1 hr.

Resin was washed 3 times with buffer A. Resin samples (referred to as eluates) and extracts were analyzed by SDS-PAGE, immuno-blotting probed with Omni-probe anti-sera (Santa Cruz), and visualised with enhanced chemiluminescence (Amersham). GSTCdc24p (1-127 aa), similar to GST, did not bind His<sub>6</sub>Ste4p. Similar results were observed in 5 independent experiments.

### C) Ste4p mutants

Ste4p is the  $\beta$ -subunit of the heterodimeric G protein that can usually associate with Cdc24p exemplified by nucleotide SEQ ID No. 9 and amino acid SEQ ID No. 10. A mutation in STE4 exemplified by nucleotide SEQ ID No. 11 and SEQ ID No. 13 and amino acid SEQ ID No. 12 and SEQ ID No. 14 prevented the interaction of the mutant G protein  $\beta$  subunit with Cdc24p. Thus, it is possible to devise assays based on this mutation to screen for agents capable of modifying the non-interactive behaviour of the mutant G protein  $\beta$  subunit with Cdc24p. In addition, the assay could be used to study Cdc24p homologues or even Cdc24p derivatives or homologues to see if those derivatives or homologues affect the non-interactive behaviour of the mutant G protein  $\beta$  subunit.

The Ste4p mutants are also aspects of the present invention.

In this regard, the present invention also covers an STE4 mutant.

The present invention also covers a mutation of the  $\beta$ -subunit of the heterodimeric G protein that can usually associate with GEF (preferably Cdc24p) that is capable of preventing the interaction of the mutant G protein subunit with GEF (preferably Cdc24p).

Hence, a further aspect of the present invention is a mutation in STE4 - i.e. on the  $\beta$ -subunit of the heterodimeric G protein that can usually associate with Cdc24p. This mutation prevents the interaction of the mutant G protein subunit with Cdc24p. Thus, likewise, it is possible to devise similar assays based on this mutation to screen for agents that modify the non-interactive behaviour of the mutant G protein with Cdc24p. In addition, the assay could be used to study Cdc24p homologues or even Cdc24p derivatives or variants to see if those

derivatives or variants affect the non-interactive behaviour of the mutant G protein. The sequences associated with this aspect of the present invention are shown as SEQ ID No. 9 etc. The present invention also covers variants or derivatives of such sequences - wherein the variants or derivatives of the wildtype sequences do not substantially affect Cdc24 interaction; and wherein the variants or derivatives of the mutant sequences do substantially affect Cdc24 interaction.

**D) Assay system to monitor the effects of two human oncogenic agents on an *S. cerevisiae* yeast mutant with a mating defect.**

An assay system was devised to establish whether two different proto-oncogenes could complement the *S. cerevisiae* yeast phenotype (*cdc24-m1*) mating defect as described above and in Nern and Arkowitz (Nature (1998) 391: 195-198). The two oncogenic agents used were the human proto-oncogene, proto-Dbl and the mouse C4 protein which is almost identical to the human sequence, C5 Vav, and which is referred to hereafter as Vav. The *S. cerevisiae* cell division cycle molecule, Cdc24p, which is a protein with similarities to proto-Dbl was used as a positive control in addition to the Cdc24p of the related yeast *K. lactis*.

Transgenic yeast organisms which co-expressed the nucleotide sequence (SEQ ID No. 3) for the *cdc24-m1* mating defect and the nucleotide sequence of interest (NOI) encoding either proto-Dbl, Vav or two related Cdc24p's were used.

The expression levels of the proto-oncogene, proto-Dbl, in *S. cerevisiae* were relatively low compared with the expression levels of the Cdc24p protein from either *S. cerevisiae* or *K. lactis*.

Qualitatively, both proto-Dbl and *K. lactis* Cdc24 proteins partially complemented the mating defect in the *cdc24-m1* mutant. This result is in contrast to that obtained with the oncogenic form of Dbl alone which, although expressed, did not complement the *cdc24-m1* mating defect. The Vav protein, did not display any effect on the mating defect. This lack of effect may be due to either insufficient expression of the Vav protein or to the fact that Vav function

requires a phosphorylation of the Lck kinase which must be co-expressed with the Vav protein before an effect can be observed.

#### E) Assays to determine FAR1 interaction with Cdc24p and G $\beta$

5

Studies have shown that *FAR1* may play an important role both for pheromone mediated growth arrest and growth orientation during mating (Valtz, N., Peter, M. & Herskowitz, I. *J. Cell Biol.* 131, 863-73 (1995); Chang, F. & Herskowitz, I. *Cell* 63, 999-1011 (1990); Peter, M., Gartner, A., Horecka, J., Ammerer, G. & Herskowitz, I. *Cell* 73, 747-60 (1993)). The orientation function, which is specifically disrupted in a *far1-H7* mutant, is required for the Cdc24 G $\beta$  interaction suggesting that Far1 might interact with Cdc24. Two-hybrid analyses show that indeed Far1 interacts with Cdc24.

While the Cdc24 G $\beta$  interaction requires the presence of *FAR1*, the Far1 Cdc24 interaction is independent of G $\beta$ , suggesting that Far1 might bind Cdc24 directly whereas Cdc24 G $\beta$  are part of a complex which include Far1. Far1 also interacts by two-hybrid assays with G $\beta$ , consistent with the notion that Cdc24, Far1, and G $\beta$  form a complex. In a diploid two-hybrid strain, in which a number of pheromone response genes are not expressed, we are unable to detect the Cdc24 G $\beta$  interaction. However, overexpression of Far1 results in an interaction and further overexpression of G $\gamma$  results in a maximal interaction, indicating that a complex comprised of Cdc24, G $\beta\gamma$ , and Far1 forms even in diploid cells.

Although *cdc24-m* and *far1-s* mutants result in similar defects in growth orientation during mating, we set out to determine if these genes function in the same orientation process. Generation of a *cdc24-m1* mutation in a  $\Delta far1$  strain did not result in a substantial decrease in mating efficiency, suggesting these two genes function in the same process. In contrast, results from double mutants of *cdc24-m1* with  $\Delta spa2$ ,  $\Delta ste20$ , or  $\Delta bem1$  suggest that these three genes do not function in the same orientation process as Cdc24 and Far1. Cdc24 and Far1 were epitope tagged in order to determine whether these proteins interact in yeast cells. The chromosomal copy of Cdc24 was replaced with a 3xmyc tagged Cdc24 and the chromosomal copy of Far1 was replaced with Far1 protein A fusion. Both of these fusion proteins are fully functional. Isolation of Far1-protein A from yeast extracts using IgG-

Sepharose co-precipitated 3xmyc-Cdc24. In contrast, the 3xmyc-Cdc24-m1 mutant was defective in binding Far1 in similar immunoprecipitation assays. These results indicate that Cdc24 and Far1 bind one-another and this interaction may be essential for growth orientation during mating.

### *Far1 binds Cdc24 and Gβ*

The binding relationships between Cdc24, Far1, and Gβ were examined *in vitro* using proteins purified from bacteria and yeast. Gβγ was purified from yeast cells using a chromosomal copy of the gene which has HA epitope (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) fused to the amino-terminus and protein A fused to the carboxyl-terminus. A tobacco etch virus (TEV) protease cleavage site (recognition site Glu-Asn-Leu-Tyr-Phe-Gln-Gly with cleavage occurring between Gln and Gly) was placed between Gβ and the protein A domain so that material isolated from yeast using IgG-Sepharose can be specifically eluted with commercially available recombinant TEV protease. Maltose binding protein (MBP) Far1 fusions have been expressed and purified from *E. coli*. Similarly, a glutathione-S-transferase (GST) Cdc24 fusion (residues 1 - 472) has been expressed and purified from *E. coli*. MBP-Far1 binds GST-Cdc24 specifically. The removal of the 75 carboxyl-terminal residues of Far1 (H7) prevents Cdc24 binding. Furthermore GST alone is unable to bind MBP-Far1.

These results show that Cdc24 can directly bind Far1 in the absence of any other yeast proteins. Far1 fragments containing either the amino-terminal Lim domain (a domain implicated in protein-protein interactions) or the carboxyl-terminus were tested for their ability to bind GST-Cdc24. Both fragments showed very little binding to GST-Cdc24 indicating that although the Far1 carboxyl-terminus is necessary, it is not sufficient for Cdc24 binding. Using MBP-Far1 we have been able to observe binding to Gβ purified from yeast. Binding of Gβ is reduced using amino-terminal or carboxy-terminal MBP-Far1 fragments, yet Gβ binds Far1H7 as well as Far1.

In one preferred aspect of the present invention the assay also includes the presence of Far1.

**RESULTS**Table 1 *cdc24-m1* is defective in cell mating

Strain	Tester	% Mating efficiency
<i>CDC24 MAT<math>\alpha</math></i>	<i>MAT<math>\alpha</math></i> WT	100 (21)
<i>cdc24-m1 MAT<math>\alpha</math></i>	<i>MAT<math>\alpha</math></i> WT	0.5 (0.2)
<i>CDC24 MAT<math>\alpha</math></i>	<i>MAT<math>\alpha</math></i> WT	100 (20)
<i>cdc24-m1 MAT<math>\alpha</math></i>	<i>MAT<math>\alpha</math></i> WT	3.8 (1.6)
<i>CDC24 MAT<math>\alpha</math></i>	<i>MAT<math>\alpha</math> <math>\Delta</math>fus1 <math>\Delta</math>fus2</i>	100(17)
<i>cdc24-m1 MAT<math>\alpha</math></i>	<i>MAT<math>\alpha</math> <math>\Delta</math>fus1 <math>\Delta</math>fus2</i>	$\leq 0.02$
<i>CDC24 MAT<math>\alpha</math></i>	<i>CDC24 MAT<math>\alpha</math></i>	100(18)
<i>cdc24-m1 MAT<math>\alpha</math></i>	<i>cdc24-m1 MAT<math>\alpha</math></i>	$\leq 0.0006$

Mating efficiencies are the number of diploid cells divided by the total cells with *CDC24* WT set to 100%. The values are means of 4 determinations with standard deviation (). Absolute mating efficiency was 14-15% with *MAT $\alpha$*  and *MAT $\alpha$*  testers, 1.8% with  $\Delta$ fus1  $\Delta$ fus2 tester, and 3.4% with *CDC24* tester.

Some of the results are also shown in the accompanying Figures. These Figures are now discussed in more detail.

## FIGURE. 1

*cdc24-m1* phenotypes. a, Actin cytoskeleton of *cdc24-m1* cells shows polarised distribution. Bar equals 5  $\mu$ m. b, Pheromone-induced growth arrest is similar in *cdc24-m1* with WT cells. Sterile filter disks spotted with  $\alpha$ -factor (1, 0.5, 0.2, 0.1, 0.05, and 0.012 mg) were placed onto cells in agarose. c, MAP-kinase pathway signalling is unaffected in *cdc24-m1*. *LacZ* activities are the average of 2 experiments (2-3 determinations per experiment) with standard deviation. WT maximum (29.6 Miller Units) was set to 100%.

## FIGURE. 2

*cdc24-m1* cells are unable to orient in a pheromone gradient. a, Excess pheromone has a negligible effect on *cdc24-m1* mating. *MATa* cells were mated with a WT tester and mating efficiency for *CDC24* (2.8%) was set to 100%. Values are means (n=2). b, *cdc24-m1* cells are unable to orient in a pheromone gradient. A trace of cell shapes after 6-7 hr in a pheromone gradient is shown with arrowheads indicating orientation. Quantitation of cell projection angle relative to the micropipet (needle) from 4-7 separate experiments (n=112 *CDC24* and 167 *cdc24-m1* cells). The average cosine of the angle of cell projection relative to the micropipet was 0.52 for *CDC24* and -0.02 for *cdc24-m1* cells (a cosine of 1 represents perfect orientation and 0, random orientation). c, *cdc24-m1* cells position their shmoo adjacent their bud scar. The position of the bud scar on zygotes was determined for approximately 120 cells.

## FIGURE. 3

*cdc24-m* mutants are defective in mating and Ste4p (G $\beta$ ) binding. a, Location of Cdc24p mating mutations. Mating patches show diploids from mating with *MATa* WT tester. Ste4 2-H patch growth on -leu-trp-his indicates an interaction of Cdc24p (1-288 aa) with Ste4p. Similar results were obtained using a *LacZ* reporter in strain Y187 (relative Miller Units 100 for Cdc24/Ste4 and 3 for Cdc24-m1/Ste4). b, Two hybrid interactions of Cdc24p. For interactions with Ste4p, a fragment of Cdc24p (1-288 aa) was used, however, full length Cdc24p also interacts with Ste4p. c, Region of Cdc24p necessary for Ste4p interaction. Numbers refer to Cdc24p aa fused to DBD. d, Cdc24p binds to Ste4p in the absence of other yeast proteins. Mixed bacterial cell

extracts (1 eq) containing either His<sub>6</sub>Ste4p and GST or GSTCdc24p (1-472 aa), and GSH-agarose eluates (800 eq) were separated by SDS-PAGE, immuno-blotted and probed with anti-sera to His<sub>6</sub>Ste4p. Anti-GST sera showed similar amounts of GST and GSTCdc24p in eluates. Due to proteolysis, His<sub>6</sub>Ste4p migrates as a doublet.

#### FIGURE. 4

Model for signal transduction pathway required for cell orientation. For clarity we have omitted components of MAP-kinase cascade. The role of Cdc42p (a Rho-family GTPase) in cell orientation is speculative. Pheromone binds the pheromone receptor (Ste2p or Ste3p) resulting in the dissociation of G $\alpha$  (Gpa1p) from G $\beta\gamma$  (Ste4p/Ste18p). Direct binding of Cdc24p to G $\beta\gamma$  (in the vicinity of the receptor) activates or recruits Cdc42p which is necessary for oriented growth towards a mating partner.

#### SEQUENCE ANALYSIS

The DH and PH sequences were analysed by a Blast homology search. In addition, an analysis of the amino acid identity over the entire protein to *S. cerevisiae* Cdc24p was conducted. DH refers to the Dbl homology region (GEF region) - see Hart *et al* 1991 Nature 354 311-314; Miyamoto *et al* 1991 Biochem Biophys Res Commun 181 604-610; Ron *et al* 1991 New Biol 3 372-379. PH refers to the Pleckstrin homology region - see Musacchio *et al* Trends Biochem Sci 18 343-348.

The results are as follows:

#### A. Blast homology search using Cdc24 DH and PH region TBLASTN 1.4.9 MP

Query= yeast Cdc24p DH PH (392 aa):

KIIKEFVATERKYVHDLEILDKYRQQLDSNLITSEELYMLFPNLGDAIDFQRRFLISLEI  
NALVEPSKQRIGALFMHSHFFKLYEPWSIGQNAAIEFLSSTLHKMRVDESQRFIINNKL  
ELQSFLYKPVQRLCRYPLLVKELLAESSDDNNTKELEAALDISKNIARSINENQRRTEN



31

HQVVKKLYGRVVNWKGYRISKFGELLYFDKVFISTTNSSEPEREFEVYLFEKIILFSE  
 VVTKKSASSLILKKKSSTSASISASNITDNNGSPHHSYHKRHSNSSSSNNIHLSSSSAAAI  
 HSSTNSSDNNSNNSSSSSLFKLSANEPKLDLRGRIMIMNLNQIIPQNNRSLNITWESIKEQ  
 GNFLKFKNEETRDNWSSCLQLIHD LKN

5

Database: Non-redundant Genbank+EMBL+DDBJ+PDB sequences

349,525 sequences; 540,957,745 total letters

Reference: Altschul, Stephen F., Warren Gish, Webb Miller, Eugene W. Myers, and David J.

10 Lipman (1990). Basic local alignment search tool. J. Mol.Biol. 215:403-410.

		Reading Frame	High Score	Smallest Sum Prob ability P(N)	Smallest Sum Prob ability N
gb U12538 SPU12538	Schizosaccharomyces pombe scd1	+3	171	1.0e-51	6
emb X57298 MMMCF2PO	M.musculus Mcf2 proto-oncogene (Mcf2 is Dbl)	+1	128	8.3e-10	3
gb U16296 HSU16296	Human T-lymphoma invasion and metastasis inducing TIAM1	+3	88	2.3e-09	3
gb U05245 MMU05245	Mus musculus BALB/c invasion inducing protein (Tiam-1)	+3	88	5.5e-09	3
gb J03639 HUMDBLTP	Human DBL oncogene encoding a transforming protein	+2	121	2.1e-07	3
gb S76992 S76992	VAV2=VAV oncogene homolog human	+3	125	2.6e-07	2
dbj D86547 D86547	Fruitfly still life type 1	+2	76	5.4e-07	5
gb U37017 MMU37017	Mus musculus Vav2 oncogene	+1	126	6.4e-07	2
dbj D86546 D86546	Fruitfly still life type 2	+1	76	1.0e-06	5
gb U39476 RNU39476	Rattus norvegicus p95 Vav proto-oncogene	+3	116	6.3e-06	1
gb S76838 S76838	Dbs (Dbl guanine nucleotide exchange factor homolog) murine	+3	112	4.4e-05	2
dbj AB002360 AB002360	Human KIAA0362	+2	113	4.5e-05	2
emb Z35654 RNOSTOG	R.norvegicus Ost oncogene	+1	112	4.9e-05	2
emb X83931 HSVAVONCO	H.sapiens VAV oncogene	+1	109	5.5e-05	1
gb AF003147 CELC11D9	Caenorhabditis elegans C11D9	+3	81	0.0070	3
gb U96634 MMU96634	Mus musculus p85SPR	+2	62	0.016	3

emb Y10159 DDY10159	D.discoideum racGAP	+1	71	0.025	3
gb U58203 MMU58203	Mus musculus Lsc oncogene	+2	75	0.044	2
emb Y09160 HSSUB15	H.sapiens Sub1.5	+1	80	0.063	2
gb AF003740 CELC41D11	Caenorhabditis elegans C41D11	+2	81	0.064	4
gb U02081 HSU02081	Human guanine nucleotide regulatory protein (NET1)	+1	77	0.12	2
gb U00055 CELR02F2	Caenorhabditis elegans R02F2	+1	85	0.13	1
gb U64105 HSU64105	Human guanine nucleotide exchange factor p115-RhoGEF	+1	77	0.14	1
gb U42390 HSU42390	Homo sapiens Trio	+1	74	0.33	3
gb M24603 HUMBCRD	Human bcr protein amino end	+1	58	0.91	3
emb X02596 HSBCRR	Human bcr (breakpoint cluster region) in Philadelphia chromosome	+3	58	0.996	3
gb U11690 HSU11690	Human faciogenital dysplasia (FGD1)	+2	73	0.999	1
gb U22325 MMU22325	Mus musculus faciogenital dysplasia (Fgd1)	+3	73	0.9997	2
gb M15025 HUMBCRABL	Human BCR/ABL product of the translocation of t(22q11; 9q34)	+3	58	0.99995	5

**B. Amino acid identity over entire protein to *S. cerevisiae* Cdc24p**

	Organism	gene	protein size (aa)	% identity (aa)
5	<hr/>			
	Schizosaccharomyces pombe	Scd1	834	21.9
	Mouse	Fgd1	960	16.7
	Human	Fgd1	961	16.5
10	Mouse	Vav2	868	16.5
	Mouse	Ect2	768	16.2
	Human	Vav2	878	15.8
	Worm	Q18479	860	15.4
	Mouse	Vav	844	14.6
15	Rat	Vav	843	14.5
	Human	Vav	846	14.4
	Mouse	Dbp	1150	14.3
	Human	Tim	519	14.0
	Human	proto-Dbp	925	13.4
20	Human	p115RhoGEF	912	13.4
	Mouse	Lfc	572	13.4
	Rat	Ost	872	12.9
	Worm	Q22354	862	12.9
	Mouse	Lsc	919	12.5
25	Human	Lbc	424	12.4
	Human	Net1	460	12.3
	Human	BCR	1271	11.9
	Mouse	Tiam1	1591	11.2
	Human	Tiam1	1591	10.9
30	Mouse	proto-Dbp	320 (partial)	9.7
	Drosophila	Still Life 1	2064	9.0
	Drosophila	Still Life 2	2044	8.4

Protein name key:

- Scd1: Schizosaccharomyce pombe Cdc24p<sup>101</sup>.
- Fgd1 Faciogenital Dysplasia Protein. FGD also known as Aarskog-Scott syndrome, is  
 5 an X-linked developmental disorder<sup>102</sup>.
- Vav/Vav2 A oncogene derived from hematopoietic cells<sup>103</sup>.
- Q18479 (similar to Vav)
- Q22354 (similar to Vav)
- Ect2 Oncogene expressed in epithelial cells and possessing transforming potential<sup>104</sup>.
- 10 Tim Mammary epithelial oncogene<sup>105</sup>.
- Dbl/Dbp Diffuse b-cell lymphoma (dbl) oncogene<sup>106, 107</sup>.
- p115RhoGEF Regulates cell proliferation, induces the transformation of cells<sup>108</sup>.
- Lfc Hematopoietic oncogene<sup>109</sup>.
- Ost Osteosarcoma derived proto-oncogene. Truncation is oncogenic and highly  
 15 tumorigenic in mice<sup>110</sup>.
- Lsc Oncoprotein<sup>111</sup>.
- Lbc Oncogene involved in chronic myeloid leukemias<sup>112</sup>.
- Net1 Neuroepithelioma transforming oncogene<sup>113</sup>.
- BCR bcr (breakpoint cluster region), an oncogene which is the translocation  
 20 breakpoint in chronic myeloid leukemias (CML)<sup>114, 115</sup>.
- Tiam1 Human invasion- and metastasis-inducing tiam1 gene and is expressed in tumor-  
 cell lines of different tissue origin<sup>116</sup>.
- Still Life 1/2 A synaptic terminal protein<sup>117</sup>.

## DISCUSSION

*CDC42* and its GDP/GTP exchange factor (GEF) *CDC24* are required for vegetative growth<sup>8,9</sup> and cell mating<sup>6,7,10</sup>. The precise function of these proteins in cell mating has been difficult to study because they are essential for viability. In accordance with the present invention, we reasoned that if *CDC24* has a specific function in the mating pathway, *cdc24* alleles should exist which affect cell mating but not vegetative growth. To identify such alleles, a collection of *CDC24* random mutants was screened and three recessive mating mutants, *cdc24-m1-3* were isolated (Figure 3A). This screen required isolated *cdc24* mutants to be able to support vegetative growth. Further characterisation of *cdc24-m* cells revealed normal growth between 18° and 37° and cell morphology, bud site selection, and actin distribution were similar to WT cells (see below and Figure 1A). The specificity of the *cdc24-m* phenotype is in contrast to that of all other described *cdc24* mutants which have strong defects in vegetative growth<sup>8-10</sup>.

To elucidate the role of *CDC24* in mating, we examined *cdc24-m1* cells for defects in the mating pathway. The mating efficiency of *cdc24-m1* cells with a WT partner was reduced approximately 100-fold compared to WT (Table 1), and this effect was essentially independent of mating type. When *cdc24-m1* or an enfeebled mate defective in cell fusion were used as mating partners, significantly stronger defects were observed. Such bilateral mating defects suggest impairment in a process such as shmoo (mating projection) formation, orientation, or fusion in which a WT mating partner can partially compensate for the mutant strain.

Pheromone activation results in a number of responses including cell cycle arrest, MAP-kinase cascade mediated induction of mating specific genes, and changes in cell morphology<sup>4,5</sup>.

Pheromone-induced growth arrest determined by halo-assays showed both *cdc24-m1* and WT cells responded similarly (Figure 1B). Furthermore, overexpression of the  $\beta$ -subunit of the yeast hetero-trimeric G-protein, Ste4p, from an inducible promoter arrested growth of both *cdc24-m1* and WT cells (data not shown). Microscopic examination revealed identical numbers of WT and *cdc24-m1* cells (78%, n=1600) formed shmoos after 4 hr exposure to 10 mM pheromone.

The actin distribution of *cdc24-m1* budding and shmooing cells was also similar to that of WT cells (Figure 1A), demonstrating that the mating defect was not due to an inability to polarise the actin cytoskeleton. The level of pheromone induced FUS1-lacZ expression, a reporter used to

measure induction of mating specific genes<sup>11</sup>, was similar in *cdc24-m1* and WT cells (Figure 1C). However, examination of mating mixtures of *cdc24-m1* and WT tester cells showed a greater than ten-fold decrease in the number of zygotes, indicating that the *cdc24-m1* defect occurs prior to cell fusion. Thus *cdc24-m* cells appear normal for cell cycle arrest, shmoo formation, actin cytoskeleton polarisation, and MAP-kinase signalling, yet are defective at a step prior to cell fusion.

During mating, polarised growth towards a mating partner requires a pheromone gradient<sup>12</sup> and saturation with pheromone during mating results in random orientation of growth and mating partner selection, and hence a decrease in mating efficiency<sup>13,14</sup>. WT cells showed a 16-fold decrease in mating efficiency in the presence of saturating pheromone (20 mM), whereas only 10% reduction was observed with *cdc24-m1* cells (Figure 2A), suggesting that this mutant is unable to orientate towards a pheromone gradient during mating. Similar results were observed with *cdc24-m2* and *cdc24-m3* cells. To test directly whether *cdc24-m1* cells are defective in mating projection orientation their response to an artificial pheromone gradient created by a micropipet was examined. While *CDC24* cells oriented growth towards the pheromone source (greater than 70% of cells oriented within 60° angle of micropipet), *cdc24-m1* cells did not show a preferred orientation (Figure 2B). No difference in the sensitivity of WT or mutant cells to pheromone was observed.

Although *cdc24-m1* cells oriented randomly in a pheromone gradient, the choice of shmoo site could be dictated by an internal cue, such as the previous bud site. To examine this possibility, the location of the bud scar (in cells with a single bud scar) relative to the neck of the zygote was determined. While WT cells showed a random position of their bud scar on the zygotes, 86% of *cdc24-m1* zygotes had formed a shmoo adjacent to their previous bud site (Figure 2C). Together these results establish a specific role for Cdc24p in orientation towards a mating partner.

Sequencing of *cdc24-m* alleles revealed mutations that changed one of two adjacent amino acid residues (Figure 3A). *cdc24-m1* and *cdc24-m3* both have a single amino acid change from Ser 189 to either a Phe or Pro. *cdc24-m2* had two amino acid substitutions and subcloning demonstrated that the mutation responsible for the mating defect is Asp to Gly at residue 190.

The grouping of these mutations suggests that this region of Cdc24p is important for an interaction required for oriented growth.

Previous two-hybrid studies have suggested that the amino-terminus of Cdc24p might interact with Ste4p<sup>7</sup>, however, the *in vivo* significance of this association was unclear. We determined whether Cdc24p mating mutants could interact with Ste4p (Figure 3A). In contrast to the wild-type Cdc24p, the mutants did not show a detectable interaction with Ste4p. In agreement with the clustering of the *cdc24-m* mutations, amino acid residues 170 to 245 of Cdc24p were sufficient for the Ste4p interaction (Figure 3C), while an amino-terminal fragment consisting of the first 160 amino acid residues, although expressed, failed to interact. Consistent with a functional significance of the Cdc24p Ste4p interaction, we have isolated mutants in *STE4*, (exemplified by SEQ ID No. 9 and SEQ ID No. 10), using a two-hybrid screen, which are unable to interact with Cdc24p and are phenotypically similar to *cdc24-m* mutants.

To assess the specificity of the defect in the interaction between Ste4p and Cdc24-mlp, interactions with Cdc42p and Bem1p, two proteins known to bind to Cdc24p<sup>15,16</sup> were investigated. Bem1p is an SH3 domain protein involved in bud formation and mating<sup>17</sup>. Cdc24-mlp was able to interact with both Cdc42p and Bem1p (Figure 3B) consistent with the absence of an effect of *cdc24-ml* on vegetative growth.

While the *cdc24-ml* phenotype along with the two-hybrid results indicates that the interaction between Cdc24p and G $\beta$  is central to cell orientation, these results do not address whether this interaction is direct or indirect. G $\beta$  typically functions as a complex with the third subunit of a hetero-trimeric G-protein, G $\gamma$ . We therefore determined whether the yeast G $\gamma$ , Ste18p, was required for the Cdc24p Ste4p interaction. Deletion of *STE18* abolished the Cdc24p Ste4p two-hybrid interaction (data not shown), suggesting that Cdc24p interacts with the G $\beta\gamma$ -complex. To determine if Cdc24p could directly bind Ste4p, these proteins were expressed in bacteria. Hexahistidine-tagged Ste4p specifically bound to GSTCdc24p (Figure 3D). These results demonstrate that Cdc24p can directly bind G $\beta$  in the absence of any other yeast proteins. We attribute the requirement for G $\gamma$  in the two-hybrid assays to its stabilisation of G $\beta$ <sup>18</sup>.

Pheromone receptor activation results in dissociation of  $G\beta\gamma$  from  $G\alpha$  at the receptor. Our results indicate that the orientation defect in *cdc24-m* cells is due to a specific defect in the Cdc24p  $G\beta\gamma$  interaction. This suggests a model in which direct binding of Cdc24p to  $G\beta\gamma$  results in recruitment (to the vicinity of the receptor) or activation of Cdc42p and that this local concentration of activated Cdc42p is responsible for oriented growth towards a pheromone gradient (Figure 4). In the absence of this recruitment or activation a site adjacent to the previous bud site appears to function as a default site for shmoo formation. Our results together with previous studies implicating Cdc24p in bud site selection<sup>8</sup>, suggest that Cdc24p acts as a crucial component required both for bud and shmoo site selection, perhaps functioning as a kind of molecular selector switch between internal signals for bud site selection and external signals for shmoo site selection. It is likely that local activation of Cdc24p recruits and activates the Rho GTPase Cdc42p, which could then interact with downstream targets required for orientation of the cytoskeleton. Cdc42p interactions with the protein kinase Ste20p<sup>19,20</sup> are not necessary for cell orientation<sup>20</sup>, suggesting that novel targets of Cdc42p are required for oriented growth towards a mating partner.

Cdc24p belongs to a diverse family of GEFs which include many mammalian proto-oncogenes<sup>2</sup>. This group of proteins shares a conserved region consisting of a Dbl-domain (named after the human proto-oncogene Dbl) followed by a pleckstrin-homology domain (PH). Sequence comparison revealed similarity between a small stretch of amino acids flanking the *cdc24* mating mutations and Dbl (Figure 3A). Our results indicate that an association between Cdc24p and  $G\beta\gamma$  links pheromone receptor activation to shmoo orientation. We propose that other GEFs, such as the proto-oncogene Dbl, provide a similar connection between G-protein coupled receptor activation and polarised cell growth.

Hence, in accordance with the present invention there are provided the following uses and utilities of Cdc24p/Ste4 interaction and *cdc24-m* mutants

- 1) Peptide inhibitors which block GEF/ $G\beta$  interaction. Peptides and peptidyl derivatives based regions encompassing mutants will be used to block and/or antagonise GEF (such as the proto-oncogenes Dbl or Vav)  $G\beta$  interaction. Derivatives of these peptides (including peptide mimics)



which bind with higher affinity will also be used. The perturbation of these interactions will be of therapeutic value for example in treatment of cancers.

2) Simple yeast based assays systems (utilising mating function and interaction reporters) will be extremely useful for high through-put screening to identify molecules perturbing this GEF/G $\beta$  interaction. In particular, the qualitative effect on mating observed with the proto-oncogene, proto-Dbl, even at low levels of expression, indicates that this type of assay is amenable to large scale screening for the effect of agents, such as proto-oncogenes, on induced defects in yeast and other host cells.

3) Similar Cdc24p/G $\beta$  interactions will be ideal targets for anti-fungal drugs directed at the pathogenic yeast *Candida*.

## SUMMARY

1) We have identified an important interaction between two general cellular components, Cdc24p and G $\beta$  which provides a connection between G protein coupled receptor activation and polarised cell growth. This work has been exemplified by work done with yeast genes/proteins, however, both cellular components involved have homologues in humans.

2) We show the physiological consequence of this interaction and from these data extrapolate to the general role of this interaction in human cells.

3) In addition, we have identified sequences required for this interaction. Specifically, we have identified a short stretch of one protein (Cdc24p) encompassing 75 aa sufficient for this interaction and three amino acid changes (within this stretch) which block the interaction and have physiological consequences. These amino acid changes fall within a 19 amino acid piece with similarity to the human proto-oncogene Dbl. Indeed, removal of this region from proto-Dbl (when the amino terminus is removed) results in oncogenicity in tissue culture cells.

4) We have also identified specific mutants in the  $\beta$ -subunit of the heterodimeric G protein (Ste4p) which appear to block its interaction with Cdc24p. We believe that several of these

mutations will fall in conserved regions of G $\beta$ . Thus, it is possible to devise assays based on this mutation to screen for agents capable of modifying the non-interactive behaviour of the mutant G protein  $\beta$  subunit with Cdc24p. In addition, the assay could be used to study Cdc24p homologues or even Cdc24p derivatives or homologues to see if those derivatives or  
5 homologues affect the non-interactive behaviour of the mutant G protein.

5) There is a wealth of information on human G $\beta$ 's, human GEF's (GDP/GTP Exchange Factors), such as Cdc24p homologues and the rho family of GTP-binding-proteins (such as rho like Cdc42p) which the GEFs work on. Most human GEF's are oncogenes such as Dbl, Vav,  
10 and Ect and are involved in some way in growth control. Furthermore G $\beta$ 's are involved in linking signals from receptors to intracellular responses. The present invention has shown that that a GEF from yeast, Cdc24p, can directly bind G $\beta$  in the absence of any other yeast proteins. Although unproven, it is likely that interactions between human GEF's and G $\beta$ 's are also crucial in growth control and chemotaxis.

15

6) We propose the interaction we have identified will have broad cellular ramifications and manipulation of these interactions (such as peptidic inhibitors and peptides mimicking activated species) will be of therapeutic value.

20 7) In addition, simple yeast based assays systems could be extremely useful for high through-put screening to identify molecules perturbing this interaction. In particular, a qualitative assay using a yeast mutant with a mating defect could prove useful in the design of agents, such as anti-cancer agents, that can affect the function of oncogenes such as proto-Dbl, in terms of its ability to complement a yeast mutant mating defect and/or its function in  
25 mammalian tissue culture cells.

8) We also believe similar interactions will be ideal targets for anti-fungal drugs directed at invasive and pathogenic yeasts such as *Candida albicans* and *Cryptococcus neoformans*.

30 All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the

41

invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular  
5 biology or related fields are intended to be within the scope of the following claims.

10

REFERENCES

1. Machesky, L.M. & Hall, A. Rho: a connection between membrane receptor signaling and the cytoskeleton. *Trends In Cell Biology* 6, 304-310 (1996).
2. Cerione, R.A. & Zheng, Y. The Dbl family of oncogenes. *Current Opinion In Cell Biology* 8, 216-222 (1996).
3. Whiteway, M., *et al.* The *STE4* and *STE18* genes of yeast encode potential  $\beta$ -subunits and  $\gamma$ -subunits of the mating factor receptor-coupled G protein. *Cell* 56, 467-477 (1989).
4. Sprague, G.F.J. & Thormer, J.W. Pheromone response and signal transduction during the mating process of *Saccharomyces cerevisiae*. In *The molecular and cellular biology of the yeast Saccharomyces* (eds. Jones, E.W., Pringle, J.R. & Broach, J.R.) 657-744 (Cold Spring Harbor Press, Cold Spring Harbor, N.Y., 1992).
5. Leberer, E., Thomas, D.Y. & Whiteway, M. Pheromone signalling and polarized morphogenesis in yeast. *Current Opinion in Genetics & Development* 7, 59-66 (1997).
6. Simon, M.-N., *et al.* Role for the Rho-family GTPase Cdc42 in yeast mating pheromone signal pathway. *Nature* 376, 702-705 (1995).
7. Zhao, Z.S., Leung, T., Manser, E. & Lim, L. Pheromone signalling in *Saccharomyces cerevisiae* requires the small GTP-binding protein Cdc42p and its activator *CDC24*. *Mol Cell Biol* 15, 5246-57 (1995).
8. Sloat, B.F., Adams, A. & Pringle, J.R. Roles of the *CDC24* gene product in cellular morphogenesis during the *Saccharomyces cerevisiae* cell cycle. *J Cell Biol* 89, 395-405 (1981).
9. Adams, A.E., Johnson, D.I., Longnecker, R.M., Sloat, B.F. & Pringle, J.R. *CDC42* and *CDC43*, two additional genes involved in budding and the establishment of cell polarity in the yeast *Saccharomyces cerevisiae*. *J Cell Biol* 111, 131-42 (1990).

10. Chenevert, J., Valtz, N. & Herskowitz, I. Identification of genes required for normal pheromone-induced cell polarization in *Saccharomyces cerevisiae*. *Genetics* 136, 1287-96 (1994).
- 5 11. Trueheart, J., Boeke, J.D. & Fink, G.R. Two genes required for cell fusion during yeast conjugation: evidence for a pheromone-induced surface protein. *Mol Cell Biol* 7, 2316-2328 (1987).
- 10 12. Segall, J.E. Polarization of yeast cells in spatial gradients of a mating factor. *Proc Natl Acad Sci U S A* 90, 8332-6 (1993).
13. Dorer, R., Pryciak, P.M. & Hartwell, L.H. *Saccharomyces cerevisiae* cells execute a default pathway to select a mate in the absence of pheromone gradients. *J Cell Biol* 131, 845-61 (1995).
- 15 14. Valtz, N., Peter, M. & Herskowitz, I. *FAR1* is required for oriented polarization of yeast cells in response to mating pheromones. *J Cell Biol* 131, 863-73 (1995).
15. Zheng, Y., Cerione, R. & Bender, A. Control of the yeast bud-site assembly GTPase Cdc42. Catalysis of guanine nucleotide exchange by Cdc24 and stimulation of GTPase activity by Bem3. *J Biol Chem* 269, 2369-72 (1994).
- 20 16. Zheng, Y., Bender, A. & Cerione, R.A. Interactions among proteins involved in bud-site selection and bud-site assembly in *Saccharomyces cerevisiae*. *J Biol Chem* 270, 626-30 (1995).
- 25 17. Chenevert, J., Corrado, K., Bender, A., Pringle, J. & Herskowitz, I. A yeast gene (*BEM1*) necessary for cell polarization whose product contains two SH3 domains. *Nature* 356, 77-9 (1992).
18. Hirschman, J.E., DeZutter, G.S., Simonds, W.F. & Jenness, D.D. The G $\beta$  complex of the yeast pheromone response pathway - subcellular fractionation and protein-protein interactions. *J Biol Chem* 272, 240-248 (1997).
- 30

19. Peter, M., Neiman, A.M., Park, H.O., Van Lohuizen, M. & Herskowitz, I. Functional analysis of the interaction between the small GTP binding protein Cdc42 and the Ste20 protein kinase in yeast. *Embo Journal* 15, 7046-7059 (1996).
- 5 20. Leberer, E., *et al.* Functional characterization of the Cdc42p binding domain of yeast Ste20p protein kinase. *Embo Journal* 16, 83-97 (1997).
21. Rose, M.D., Winston, F. & Hieter, P. *Methods in yeast genetics: a laboratory course manual* (Cold Spring Harbor Press, Cold Spring Harbor, N.Y., 1991).
- 10 22. Miyamoto, S., *et al.* A DBL-homologous region of the yeast *CLS4/CDC24* gene product is important for Ca(2+)-modulated bud assembly. *Biochem Biophys Res Commun* 181, 604-10 (1991).
- 15 23. Mitchell, D.A., Marshall, T.K. & Deschenes, R.J. Vectors for the inducible overexpression of glutathione-S-transferase fusion proteins in yeast. *Yeast* 9, 715-722 (1993).
24. Wach, A., Brachat, A., Alberti-Segui, C., Rebischung, C. & Philippsen, P. Heterologous *HIS3* marker and GFP reporter modules for PCR-targeting in *Saccharomyces cerevisiae*. *Yeast* 13, 1065-1075 (1997).
- 20 25. Fromant, M., Blanquet, S. & Plateau, P. Direct random mutagenesis of gene-sized DNA fragments using polymerase chain-reaction. *Analytical Biochemistry* 224, 347-353 (1995).
- 25 26. Sprague, G.F. Assay of yeast mating reaction. *Methods In Enzymol* 194, 77-93 (1991).
27. Adams, A.E. & Pringle, J.R. Staining of actin with fluorochrome-conjugated phalloidin. *Methods Enzymol* 194, 729-31 (1991).
- 30 28. Pringle, J.R. Staining of bud scars and other cell wall chitin with calcofluor. *Methods Enzymol* 194, 732-5 (1991).

29. Langle-Ronault, F. & Jacobs, E. A method for performing precise alterations in the yeast genome using a recyclable selectable marker. *Nucleic Acids Research* 23, 3079-3081 (1995).
30. James, P., Halladay, J. & Craig, E.A. Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics* 144, 1425-1436 (1996).
101. Chang, E.C., Barr, M., Wang, Y., Jung, V., Xu, H.P., and Wigler, M.H. 1994. Cooperative interaction of *S. pombe* proteins required for mating and morphogenesis. *Cell*. 79: 131-41.
102. Pasteris, N.G., Cadle, A., Logie, L.J., Porteous, M., Schwartz, C.E., Stevenson, R.E., Glover, T.W., Wilroy, R.S., and Gorski, J.L. 1994. Isolation and characterization of the faciogenital dysplasia (aarskog-scott syndrome) gene - a putative Rho/rac guanine-nucleotide exchange factor. *Cell*. 79: 669-678.
103. Katzav, S., Martinzanca, D., and Barbacid, M. 1989. Vav, a novel human oncogene derived from a locus ubiquitously expressed in hematopoietic-cells. *Embo Journal*. 8: 2283-2290.
104. Miki, T., Smith, C.L., Long, J.E., Eva, A., and Fleming, T.P. 1993. Oncogene *ect2* is related to regulators of small GTP-binding proteins. *Nature*. 462-465.
105. Chan, A.M., McGovern, E.S., Catalano, G., Fleming, T.P., and Miki, T. 1994. Expression cDNA cloning of a novel oncogene with sequence similarity to regulators of small GTP-binding proteins. *Oncogene*. 9: 1057-63.
106. Eva, A. and Aaronson, S.A. 1985. Isolation of a new human oncogene from a diffuse B-cell lymphoma. *Nature*. 316: 273-275.
107. Ron, D., Tronick, S.R., Aaronson, S.A., and Eva, A. 1988. Molecular-cloning and characterization of the human *dbl* proto- oncogene - evidence that its overexpression is sufficient to transform nih/3t3 cells. *Embo Journal*. 7: 2465-2473.

108. Hart, M.J., Sharma, S., Elmasry, N., Qiu, R.G., McCabe, P., Polakis, P., and Bollag, G. 1996. Identification of a novel guanine-nucleotide exchange factor for the Rho-gtpase. *Journal Of Biological Chemistry*. 271: 25452-25458.
- 5 109. Whitehead, I., Kirk, H., Tognon, C., Trigo-Gonzalez, G., and Kay, R. 1995. Expression cloning of lfc, a novel oncogene with structural similarities to guanine nucleotide exchange factors and to the regulatory region of protein kinase C. *J Biol Chem*. 270: 18388-95.
- 10 110. Horii, Y., Beeler, J.F., Sakaguchi, K., Tachibana, M., and Miki, T. 1994. A novel oncogene, ost, encodes a guanine nucleotide exchange factor that potentially links Rho and Rac signaling pathways. *Embo J*. 13: 4776-86.
- 15 111. Glaven, J.A., Whitehead, I.P., Nomanbhoy, T., Kay, R., and Cerione, R.A. 1996. Lfc and lsc oncoproteins represent 2 new guanine-nucleotide exchange factors for the Rho-gtp-binding protein. *Journal Of Biological Chemistry*. 271: 27374-27381.
112. Toksoz, D. and Williams, D.A. 1994. Novel human oncogene ibc detected by transfection with distinct homology regions to signal-transduction products. *Oncogene*. 621-628.
- 20 113. Chan, A., Takai, S., Yamada, K., and Miki, T. 1996. Isolation of a novel oncogene, net1, from neuroepithelioma cells by expression cDNA cloning. *Oncogene*. 12: 1259-1266.
114. Hariharan, I.K. and Adams, J.M. 1987. Cdna sequence for human-bcr, the gene that translocates to the abl- oncogene in chronic myeloid-leukemia. *Embo Journal*. 6: 115-119.
- 25 115. Diekmann, D., Brill, S., Garrett, M.D., Totty, N., Hsuan, J., Monfries, C., Hall, C., Lim, L., and Hall, A. 1991. Bcr encodes a gtpase-activating protein for p21rac. *Nature*. 651: 400-402.
- 30 116. Habets, G., Vanderkammen, R.A., Stam, J.C., Michiels, F., and Collard, J.G. 1995. Sequence of the human invasion-inducing tiam1 gene, its conservation in evolution and its expression in tumor-cell lines of different tissue origin. *Oncogene*. 1371-1376.



117. Sone, M., Hoshino, M., Suzuki, E., Kuroda, S., Kaibuchi, K., Nakagoshi, H., Saigo, K., Nabeshima, Y., and Hama, C. 1997. Still life, a protein in synaptic terminals of *Drosophila* homologous to GDP-GTP exchangers. *Science*. 275: 543-547.

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>18</u> , line <u>5</u>	
B. IDENTIFICATION OF DEPOSIT <span style="float: right;">Further deposits are identified on an additional sheet: <input type="checkbox"/></span>	
Name of depositary institution The National Collections of Industrial and Marine Bacteria Limited (NCIMB)	
Address of depositary institution (including postal code and country) 23 St Machar Drive Aberdeen AB2 1RY United Kingdom	
Date of deposit: 3 October 1997	Accession Number: NCIMB 40898
C. ADDITIONAL INDICATIONS (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet: <input type="checkbox"/></span>	
In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will only be made available either until the publication of the mention of the grant of the patent or after twenty years from the date of filing if the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g. "Accession Number of Deposit")	
For receiving Office use only <input checked="" type="checkbox"/> This sheet was received with the international application Authorized officer:	For International Bureau use only <input type="checkbox"/> This sheet was received by the International Bureau on: Authorized officer:

Form PCT/RO/134 (July 1992)

SUBSTITUTE SHEET (RULE 26)

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>18</u> line <u>7</u>	
B. IDENTIFICATION OF DEPOSIT <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution The National Collections of Industrial and Marine Bacteria Limited (NCIMB)	
Address of depositary institution (including postal code and country) 23 St Machar Drive Aberdeen AB2 1RY United Kingdom	
Date of deposit <u>3 October 1997</u>	Accession Number <u>NCIMB 40899</u>
C. ADDITIONAL INDICATIONS (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will only be made available either until the publication of the mention of the grant of the patent or after twenty years from the date of filing if the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g. "Accession Number of Deposit")	

<div style="border: 1px solid black; padding: 5px;">           For receiving Office use only  <input checked="" type="checkbox"/> This sheet was received with the international application  <hr/>           Authorized officer:         </div>	<div style="border: 1px solid black; padding: 5px;">           For International Bureau use only  <input type="checkbox"/> This sheet was received by the International Bureau on:  <hr/>           Authorized officer:         </div>
--	--

Form PCT/RO/114 (July 1992)

SUBSTITUTE SHEET (RULE 26)

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>18</u> , line <u>9</u>	
<b>B. IDENTIFICATION OF DEPOSIT</b> <div style="text-align: right;">Further deposits are identified on an additional sheet: <input type="checkbox"/></div>	
Name of depositary institution  The National Collections of Industrial and Marine Bacteria Limited (NCIMB)	
Address of depositary institution (including postal code and country) 23 St Machar Drive Aberdeen AB2 1RY United Kingdom	
Date of deposit  3 October 1997	Accession Number  NCIMB 40900
<b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable) <div style="text-align: right;">This information is continued on an additional sheet: <input type="checkbox"/></div>	
In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will only be made available either until the publication of the mention of the grant of the patent or after twenty years from the date of filing if the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)	
<b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)	
<b>E. SEPARATE FURNISHING OF INDICATIONS</b> (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
<div style="text-align: right; font-size: small;">For receiving Office use only</div> <div style="border: 1px solid black; padding: 5px;"><input checked="" type="checkbox"/> This sheet was received with the international application</div> <div style="border: 1px solid black; padding: 5px; margin-top: 5px;">Authorized officer</div>	<div style="text-align: right; font-size: small;">For International Bureau use only</div> <div style="border: 1px solid black; padding: 5px;"><input type="checkbox"/> This sheet was received by the International Bureau on:</div> <div style="border: 1px solid black; padding: 5px; margin-top: 5px;">Authorized officer</div>

Form PCT/RO/134 (July 1992)

SUBSTITUTE SHEET (RULE 26)

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description  
on page 18 line 11

## B. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet ☐

Name of depositary institution

The National Collections of Industrial and Marine Bacteria Limited (NCIMB)

Address of depositary institution (including postal code and country)

23 St Machar Drive

Aberdeen

AB2 1RY

United Kingdom

Date of deposit

3 October 1997

Accession Number

NCIMB 40901

## C. ADDITIONAL INDICATIONS (leave blank if not applicable)

This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will only be made available either until the publication of the mention of the grant of the patent or after twenty years from the date of filing if the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

## D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

## E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g. "Accession Number of Deposit")

For receiving Office use only

☒ This sheet was received with the international application

Authorized officer

For International Bureau use only

☐ This sheet was received by the International Bureau on:

Authorized officer

CLAIMS

1. A nucleotide sequence shown as SEQ I.D. No. 1 or a derivative, fragment, variant or  
homologue thereof, wherein the expression product of the nucleotide sequence has the  
5 capability of not substantially affecting the interaction of G $\beta$  with Cdc24p or a homologue  
thereof that is usually capable of being associated therewith.
2. A mutant of the nucleotide sequence shown as SEQ I.D. No. 1 or a derivative, fragment,  
variant or homologue thereof, wherein the expression product of the mutant nucleotide  
10 sequence has the capability of substantially affecting the interaction of G $\beta$  with Cdc24p or a  
homologue thereof that is usually capable of being associated therewith.
3. A nucleotide sequence shown as SEQ I.D. No. 1 or a derivative, fragment, variant or  
homologue thereof or the expression product thereof for use in medicine.
- 15 4. A mutant of the nucleotide sequence shown as SEQ I.D. No. 1 or a derivative, fragment,  
variant or homologue thereof or the expression product thereof for use in medicine.
5. Use of a nucleotide sequence shown as SEQ I.D. No. 1 or a derivative, fragment, variant or  
20 homologue thereof or the expression product thereof in the manufacture of a medicament to  
affect the growth behaviour of cells.
6. Use of a mutant of a nucleotide sequence shown as SEQ I.D. No. 1 or a derivative, fragment,  
variant or homologue thereof or the expression product thereof in the manufacture of a  
25 medicament to affect the growth behaviour of cells.
7. Use of a nucleotide sequence shown as SEQ I.D. No. 1 or a derivative, fragment, variant or  
homologue thereof or the expression product thereof in a screen to identify one or more agents  
that are capable of affecting the interaction of Cdc24p or a homologue thereof with a G $\beta$  or an  
30 associated Rho-family GTPase.

8. Use of a mutant of a nucleotide sequence shown as SEQ I.D. No. 1 or a derivative, fragment, variant or homologue thereof or the expression product thereof in a screen to identify one or more agents that are capable of affecting the interaction of Cdc24p or a homologue thereof with a G $\beta$  or an associated Rho-family GTPase.

9. An assay comprising contacting an agent with a nucleotide sequence shown as SEQ I.D. No. 1 or a derivative, fragment, variant or homologue thereof or the expression product thereof in the presence of a G $\beta$  capable of being associated with Cdc24p or a homologue thereof; and determining whether the agent is capable of affecting the interaction of the nucleotide sequence or the expression product with the G $\beta$ .

10. An assay comprising contacting an agent with a mutant of a nucleotide sequence shown as SEQ I.D. No. 1 or a derivative, fragment, variant or homologue thereof or the expression product thereof in the presence of a G $\beta$  capable of being associated with Cdc24p or a homologue thereof; and determining whether the agent is capable of affecting the interaction of the mutant nucleotide sequence or the expression product with the G $\beta$ .

11. A kit comprising a nucleotide sequence shown as SEQ I.D. No. 1 or a derivative, fragment, variant or homologue thereof or the expression product thereof; and a G $\beta$  capable of being associated with Cdc24p or a homologue thereof.

12. A kit comprising a mutant of a nucleotide sequence shown as SEQ I.D. No. 1 or a derivative, fragment, variant or homologue thereof or the expression product thereof; and a G $\beta$  capable of being associated with Cdc24p or a homologue thereof.

13. A protein sequence shown as SEQ I.D. No. 2 or a derivative, fragment, variant or homologue thereof, wherein the protein has the capability of not substantially affecting the interaction of G $\beta$  with Cdc24p or a homologue thereof that is usually capable of being associated with the Cdc24p or the homologue thereof.

14. A mutant of the protein sequence shown as SEQ I.D. No. 1 or a derivative, fragment, variant or homologue thereof, wherein the mutant protein has the capability of substantially

affecting the interaction of G $\beta$  with Cdc24p or a homologue thereof that is usually capable of being associated with the Cdc24p or the homologue thereof.

15. A protein sequence shown as SEQ I.D. No. 2 or a derivative, fragment, variant or  
5 homologue thereof for use in medicine.

16. A mutant of the protein sequence shown as SEQ I.D. No. 2 or a derivative, fragment, variant or homologue thereof for use in medicine.

10 17. Use of a protein sequence shown as SEQ I.D. No. 2 or a derivative, fragment, variant or homologue thereof in the manufacture of a medicament to affect the growth behaviour of cells.

15 18. Use of a mutant of a protein sequence shown as SEQ I.D. No. 2 or a derivative, fragment, variant or homologue thereof in the manufacture of a medicament to affect the growth behaviour of cells.

19. Use of a protein sequence shown as SEQ I.D. No. 2 or a derivative, fragment, variant or homologue thereof in a screen to identify one or more agents that are capable of affecting the  
20 interaction of Cdc24p or a homologue thereof thereof with a G $\beta$  or an associated Rho-family GTPase.

20. Use of a mutant of a protein sequence shown as SEQ I.D. No. 2 or a derivative, fragment, variant or homologue thereof in a screen to identify one or more agents that are capable of  
25 affecting the interaction of Cdc24p or a homologue thereof with a G $\beta$  or an associated Rho-family GTPase.

21. An assay comprising contacting an agent with a protein sequence shown as SEQ I.D. No. 2 or a derivative, fragment, variant or homologue thereof in the presence of a G $\beta$  capable of  
30 being associated with Cdc24p or a homologue thereof; and determining whether the agent is capable of affecting the interaction of the protein sequence with the G $\beta$  or the Rho-family GTPase.



22. An assay comprising contacting an agent with a mutant of a protein sequence shown as SEQ I.D. No. 2 or a derivative, fragment, variant or homologue thereof in the presence of G $\beta$  capable of being associated with Cdc24p or a homologue thereof; and determining whether the agent is capable of affecting the interaction of the mutant protein sequence with the G $\beta$  or the Rho-family GTPase.
23. A kit comprising a protein sequence shown as SEQ I.D. No. 2 or a derivative, fragment, variant or homologue thereof; and a G $\beta$  capable of being associated with Cdc24p or a homologue thereof.
24. A kit comprising a mutant of a protein sequence shown as SEQ I.D. No. 2 or a derivative, fragment, variant or homologue thereof; and a G $\beta$  capable of being associated with Cdc24p or a homologue thereof.
25. A GEF capable of interacting with a G $\beta$  such that the interaction provides a connection between G protein coupled receptor activation and polarised cell growth.
26. An agent capable of affecting a GEF/G $\beta$  interaction, which interaction provides a connection between G protein coupled receptor activation and polarised cell growth.
27. A sequence selected from: SEQ ID No. 15 or SEQ ID No. 16 or SEQ ID No. 17 or SEQ ID No. 18 or SEQ ID No. 19.
28. An assay method comprising the use of the sequence presented in claim 28 or a nucleotide sequence coding for same.
29. Use of an agent identified by the assay of claim 9 or claim 10 or claim 21 or claim 22 or claim 28 in the manufacture of a medicament which affects cell growth.

1/4

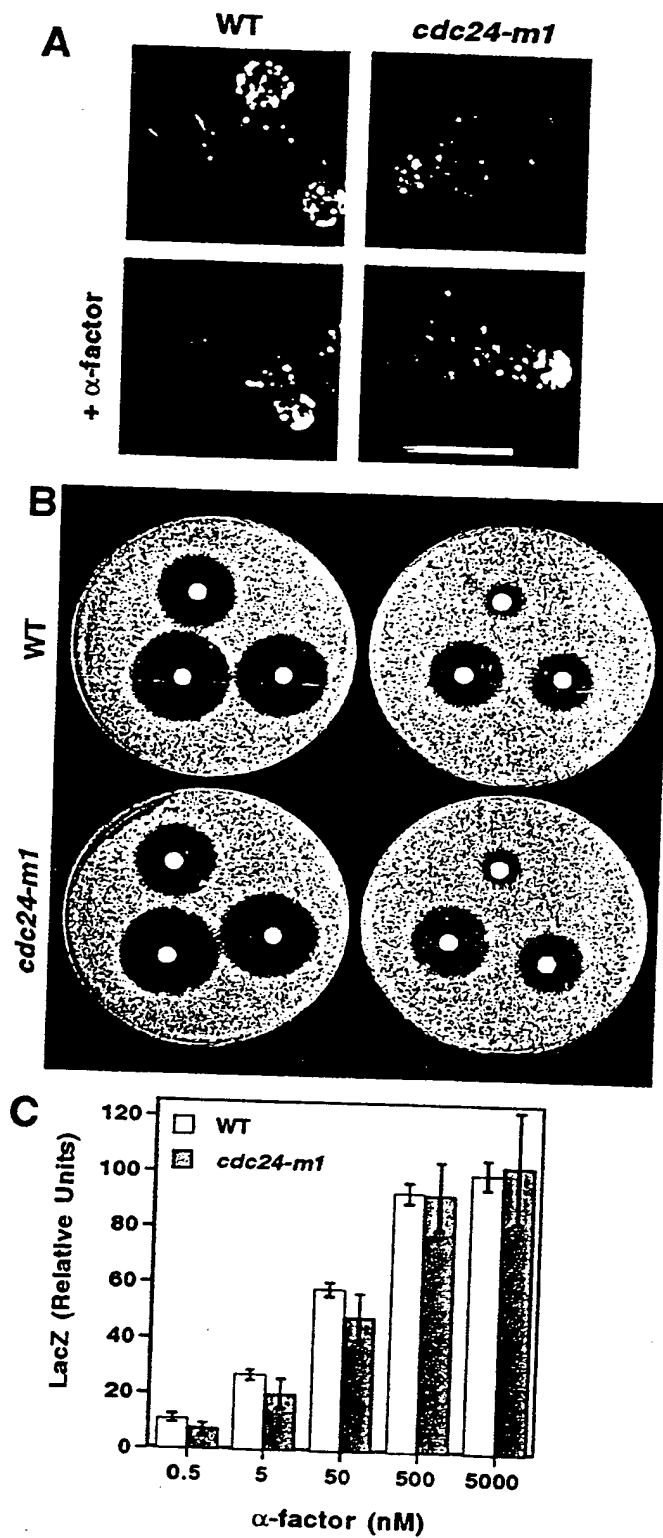


FIG. 1

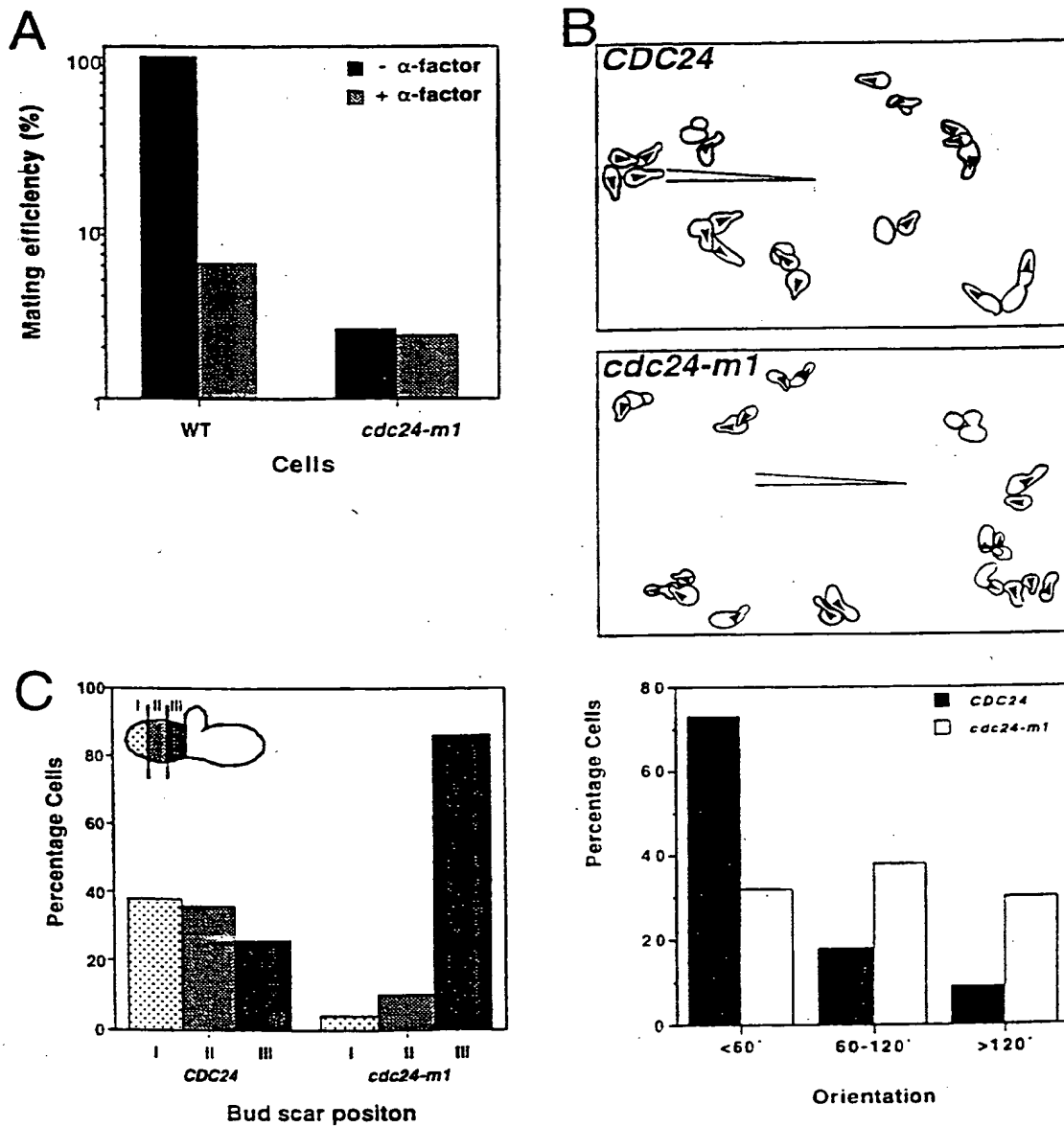
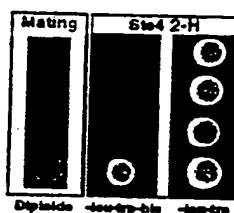


FIG. 2

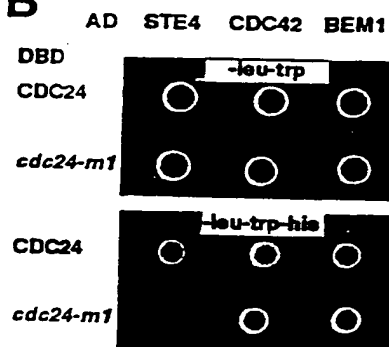
3/4

**A**

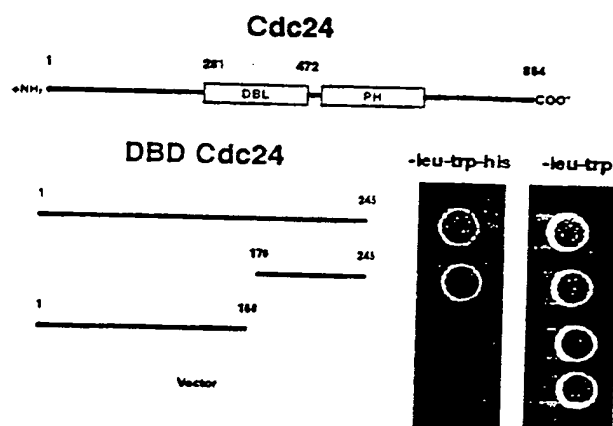
<i>Cdc24-m1</i>			QFKLPVIAFDLKVCKKSI	
<i>Cdc24-m2</i>			QFKLPVIASGLKVCKKSI	
<i>Cdc24-m3</i>			QFKLPVIAFDLKVCKKSI	
<i>Cdc24</i>	Sc	181	QFKLPVIAFDLKVCKKSI	199
			...   ...	
<i>Dbl</i>	Hu	385	QYEFDVILSPFLKVQMKTI	403



**B**



**C**



**D**

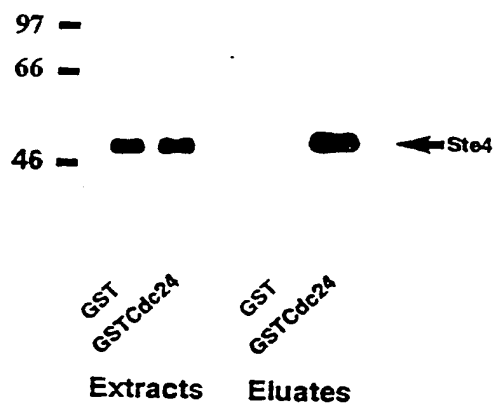


FIG. 3

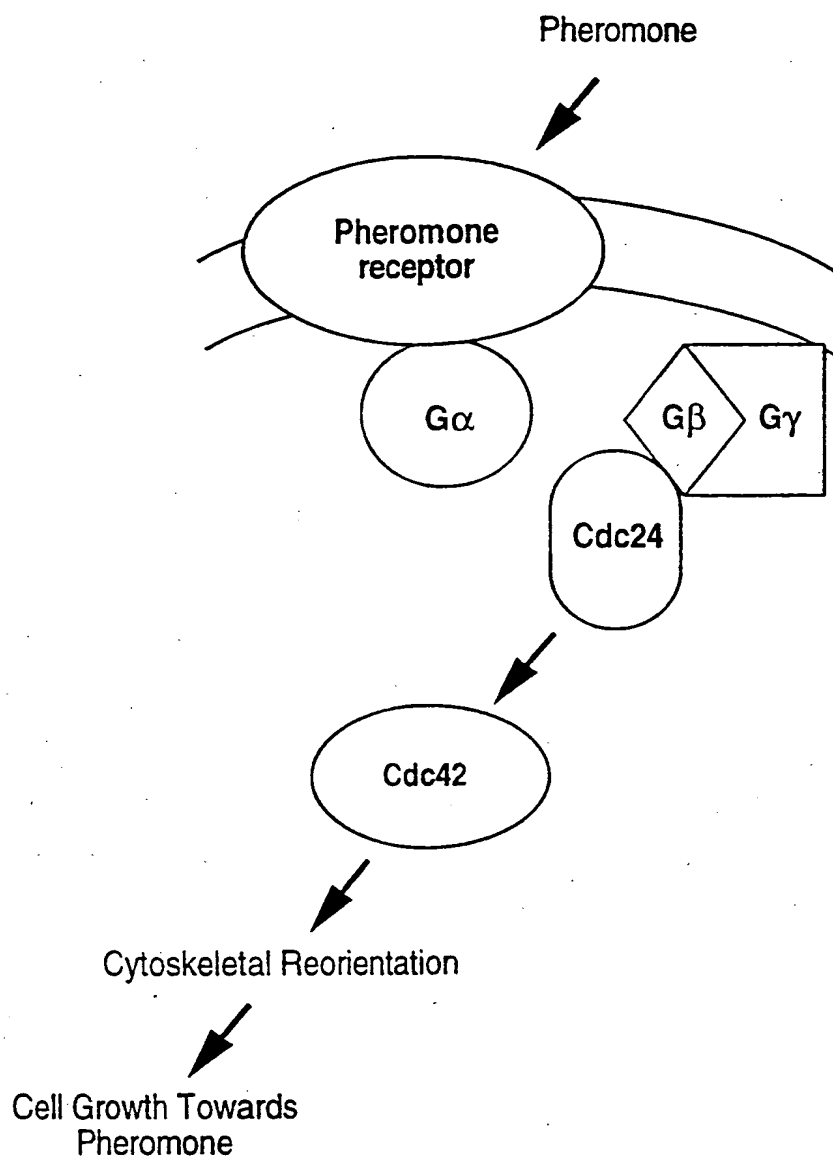


FIG. 4

SEQUENCE LISTINGSA. *cdc24* (wt)

SEQ ID NO. 1

5 DNA:

cccctctgtatacttttcaactctgtgaagccgcaatttaaattaccggtaatagcatctgacgattgaaagtctgtaaaaatccatttatgactt  
tatattgggctgcaagaaacactttgcatttaacgatgaggagcttttcactatatccgacgttttgccaactcgacgtcccagctgggtcaaag  
tgctagaagtagtagaaaacgctaataatgaattccagc

10

SEQ ID NO. 2

Protein:

PLCILFNSVKPQFKLPVIASDDLKVCKKSIYDFILGCKKHFAFNDEELFTISDVFANSTSQ  
LVKVLEVVELTMNSS

15

B. *cdc24-m1*

SEQ ID NO. 3

DNA:

20 cccctctgtatacttttcaactctgtgaagccgcaatttaaattaccggtaatagcatttgacgattgaaagtctgtaaaaatccatttatgactt  
tatattgggctgcaagaaacactttgcatttaacgatgaggagcttttcactatatccgacgttttgccaactcgacgtcccagctgggtcaaag  
tgctagaagtagtagaaaacgctaataatgaattccagc

SEQ ID NO. 4

25 Protein:

PLCILFNSVKPQFKLPVIAFDDLKVCKKSIYDFILGCKKHFAFNDEELFTISDVFANSTSQ  
LVKVLEVVELTMNSS

**C. *cdc24-m2***

SEQ ID NO. 5

DNA:

5

ccccctctgtatacttttcaactctgtgaagccgcaatttaaattaccggtaatagcatctggcgatttgaaagtctgtaaaaatccattatgactt  
tatattgggctgcaagaaacactttgcatttaacgatgaggagcttttactatatccgacgttttgccaactcgacgtcccagctgggtcaaag  
tgctagaagtagtagaaacgctaataattccagc

10 SEQ ID NO. 6

Protein:

PLCILFNSVKPQFKLPVIASGDLKVCKKSIYDFILGCKKHFAFNDEELFTISDVFANSTSQ  
LVKVLEV VETLMNSS

15

**D. *cdc24-m3***

SEQ ID NO. 7

DNA:

20

ccccctctgtatacttttcaactctgtgaagccgcaatttaaattaccggtaatagcacctgacgatttgaaagtctgtaaaaatccattatgact  
ttatattgggctgcaagaaacactttgcatttaacgatgaggagcttttactatatccgacgttttgccaactcgacgtcccagctgggtcaa  
gtgctagaagtagtagaaacgctaataattccagc

25 SEQ ID NO. 8

Protein:

PLCILFNSVKPQFKLPVIAPDDLKVCKKSIYDFILGCKKHFAFNDEELFTISDVFANSTSQ  
LVKVLEV VETLMNSS

30

SEQ ID NO. 9

*STE4* DNA sequence (wild-type)

ATGGCACATCAGATGGACTCGATAACGTATTCTAATAATGTCAACCAACAGTATATACAACCAACAGTCTACAGGA  
TATCTCTGCACTGGAGGAAGAAATTCAAATAAAATAGAGGGCCGCCAGACAAGAGAGTAAACAGCTTCATGCTCAAATAA  
ATAAAGCAAAACACAAGATACAAGATGCAAGCTTATTCCAGATGGCCAACAAAGTTACTTCGTTGACCAAAAATAAGATC  
5 AACTTAAAGCCAAATATCGTGTGAAAGGCCATAATAATAAAATCTCAGATTTTCGGTGGAGTCGAGATTCAAACGTAT  
TTTGAGTGCAAGTCAAGATGGCTTTATGCTTATATGGGACAGTGTCTCAGGTTTAAACAGAACGCTATTCCATTAGATT  
CTCAATGGGTTCTTTCTGCGCTATTTGCGCATCGAGTACTTTGGTAGCAAGCGCAGGATTAAACAATAAAGTGTACCATT  
TATAGAGTTTCGAAAGAAAACAGAGTAGCGCAAAACGTTGCGTCAATTTTCAAAGGACATACTTGCTATATTTCTGACAT  
TGAATTTACAGATAACGCACATATATTGACAGCAAGTGGGGATATGACATGTGCCTTGTGGGATATACCGAAAGCAAAGA  
10 GGGTGAGAGAAATATTCTGACCATTAGGTGATGTTTTGGCATTAGCTATTCTGAAAGAGCCAACTTAGAAAATCTCTCG  
AACACATTGCTAGCTGTGGATCAGACGGGTATACTTACATATGGGATAGCAGATCTCCGTCCGCTGTACAAAGCTTTTA  
CGTTAAGCATCTGATATTAAAGCACTTCGTTTCTTCAAAGACGCGATCTCGATTCTTCAAGCACTGACATCTGCGA  
TAAATATGATGATTTAAGGTGGGACTGTTCTATTGCTACTTTTCTCTTTTTCGAGGTTATGAAGAAAGTACCCCTACC  
CCTACTTATATGGCAGCTAACATGGAGTACAATACCGCGCAATCGCCACAACTTTAAATCAACAAGCTCAAGCTATCT  
15 AGACAACCAAGCGCTTCTTTCTTTAGATTTTAGTGCATCTGGAAGATTGATGTAATCTATACAGACATTGGTTGTG  
TTTGTGTGGGATGATTAAGAGGAGAGATTGTTGGAAATTAGAAGGTCATGGTGGCAGAGTCACTGGTGTGCGCTCGAGT  
CCAGATGGGTAGCTGTATGTACAGGTTTATGGGACTCAACCATGAAAATATGCTCTCCAGGTTATCAATAG

## 20 SEQ ID No. 10

Ste4 Protein sequence (wild-type)

MAHQMDSITYSNNVTQQYIQPQSLQDISAVEEEIQNKIEPARQESKQLHAQINKAKHKIQDASLFQMANKVTSITKNKIN  
LKPNIVLKGHNKISDFRWSRDSKRILSASQDGFMLIWDSASGLKQNAIPLDSQWVLSCAISPSTLVASAGLNNNCTIY  
25 RVSKENRVAQNVASIFKGHTCYISDIEFTDNAHILTASGDMTCALWDIPKAKRVREYSDHLGCVLALAIPEEPNLENSN  
TFASCGSDGYTYIWDSRSPSAVQSFYVNDSDINALRFFKDGMSIVAGSDNGAINMYDLRSDCIATFSLFRGYEERTFTF  
TYMAANMEYNTAQSPQTLKSTSSSYLDNQGVVSLDFSASGRMLMYSCYTDIGCVVWDVLKGEIVGKLEGHGGRVTGVRSSP  
DGLAVCTGSHWSTMKIWSPGYQ

30

## SEQ ID No. 11

*ste4-o15* DNA sequence (mutant)

ATGGCACATCAGATGGACTCGATAACGTATTCTAATAATGTCAACCAACAGTATATACAACCAACAGTCTACAGGA  
35 TATCTCTGCACTGGAGGAAGAAATTCAAATAAAATAGAGGGCCGCCAGACAAGAGAGTAAACAGCTTCATGCTCAAATAA  
ATAAAGCAAAACACAAGATACAAGATGCAAGCTTATTCCAGATGGCCAACAAAGTTACTTCGTTGACCAAAAATAAGATC  
AACTTAAAGCCAAATATCGTGTGAAAGGCCATAATAATAAAATCTCAGATTTTCGGTGGAGTCGAGATTCAAACGTAT



TTTGAGTGCAAGTCAAGATGGCTTTATGCTTATATGGGACAGTGCTTCAGGTTTAAAACAGAACGCTATTCCATTAGATT  
CTCAATGGGTTCTTTCTGCGCTATTTGCGCCATCGAGTACTTTGGTAGCAAGCGCAGGATTAAACATAACTGTACCATT  
TATAGAGTTTCGAAAGAAAACAGAGTAGCGCAAAACGTTGCGTCAATTTTCAAAGGACATACTTGCTATATTTCTGACAT  
TGAATTTACAGATAACGCACATATATTGACAGCAAGTGGGGATATGACATGTGCCTTGTGGGATATACCGAAAGCAAAGA  
5 GGGTGAGAGGATATTCTGACCATTTAGGTGATGTTTTGGCATTAGCTATTCCTGAAGAGCCAACTTAGAAAATTCTTCG  
AACACATTCGCTAGCTGTGGATCAGACGGGTATACTTACATATGGGATAGCAGATCTCCGTCGCTGTACAAAGCTTTTA  
CGTTAACGATAGTGATATTAATGCACTTCGTTTTTTCAAAGACGGGATGTCGATTGTTGCAGGAAGTGACAATGGTGCGA  
TAAATATGTATGATTTAAGGTCGGACTGTTCTATTGCTACTTTTTCTCTTTTCGAGGTTATGAAGAAGCTACCCCTACC  
CCTACTTATATGGCAGCTAACATGGAGTACAATACCGCGCAATCGCCACAACTTTAAATCAACAAGCTCAAGCTATCT  
10 AGACAACCAAGGCGTTGTTTCTTTAGATTTTAGTGATCTGGAAGATTGATGTACTCATGCTATACAGACATTGGTTGTG  
TTGTGTGGGATGTATTAAGGAGAGATTGTTGGAAATTAGAAGGTCATGGTGGCAGAGTCACTGGTGTGCGCTCGAGT  
CCAGATGGCTTAGCTGTATGTACAGGTTTCATGGGACTCAACCATGAAATATGGTCTCCAGGTTATCAATAG

## SEQ ID No. 12

15 Ste4-o15 Protein sequence (mutant)

MAHQMDSITYSNNVTQQYIQPQSLQDISAVEEEIQNKIEAARQESKQLHAQINKAKHKIQDASLFQMANKVTSLTKNKIN  
LKPNIIVLKGNNKISDFRWSRDSKRILSASQDGFMLIWDSASGLKQNAIFLDSQWVLSCAISPSSTLVASAGLNNNCTIY  
RVSKENRVAQNVAIFKGHTCYISDIEFTDNAHILTASGDMTCALWDIPKAKRVRGYSDHLGDVLAALAIPEEPNLENSN  
20 TFASCGSDGYTYIWDSRSPSAVQSFYVNDSDINALRFFKDGMSIVAGSDNGAINMYDLRSDCSIATFSLFRGYEERTPTP  
TYMAANMEYNQAQSPQTLKSTSSSYLDNQGVVSLDFSASGRMLMYSCYTDIGCVVWDVLKGEIVGKLEGHGGRVTGVRSSP  
DGLAVCTGSWDSTMKIWSPGYQ

## SEQ ID NO. 13

25 *ste4-o17* DNA sequence (mutant)

ATGGCACATCAGATGGACTCGATAACGTATTCTAATAATGTCACCCAACAGTATATACAACCACAAAGTCTACAGGA  
TATCTCTGCAGTGGAGGAAGAAATTCAAATAAAATAGAGGCCGCCAGACAGAGAGTAACAGCTTCATGCTCAATAA  
ATAAAGCAAACACAAGATACAAGATGCAAGCTTATTCAGATGGCCAACAAAGTTACTTCGTTGACCAAAAATAAGATC  
30 AACTTAAAGCCAAATATCGTGTTGAAAGGCCATAATAATAAAATCTCAGATTTTCGGTGGAGTCGAGATTCAAACGTAT  
TTTGAGTGCAAGTCAAGATGGCTTTATGCTTATATGGGACAGTGCTTCAGGTTTAAAACAGAACGCTATTCCATTAGATT  
CTCAATGGGTTCTTTCTGCGCTATTTGCGCCATCGAGTACTTTGGTAGCAAGCGCAGGATTAAACATAACTGTACCATT  
TATAGAGTTTCGAAAGAAAACAGAGTAGCGCAAAACGTTGCGTCAATTTTCAAAGGACATACTTGCTATATTTCTGACAT  
TGAATTTACAGATAACGCACATATATTGACAGCAAGTGGGGATATGACATGTGCCTTGTGGGATATACCGAAAGCAAAGA  
35 GGGTGAGAGAATATTCTGACCATTTAGGTGATGTTTTGGCATTAGCTATTCCTGAAGAGCCAACTTAGAAAATTCTTCG  
AACACATTCGCTAGCTGTGGATCAGACGGGTATACTTACATATGGGATAGCAGATCTCCGTCGCTGTACAAAGCTTTTA  
CGTTAACGATAGTGATATTAATGCACTTCGTTTTTTCAAAGACGGGATGTCGATTGTTGCAGGAAGTGACAATGGTGCGA

TAAATATGTATGATTTAAGGTCGGACTGTTCTATTGCTACTTTTTCTCTTTTTTCGAGGTTATGAAGAACGTACCCCTACC  
CCTACTTATATGGCAGCTAACATGGAGTACAATACCGCGCAATCGCCACAACTTTAAAATCAACAAAGCTCAAGCTATCT  
AGACAACCAAGGCGCTGTTTCTTTAGATTTTAGTGCATCTGGAAGATTGATGTACTCATGCTATACAGACATTGGTTGTG  
TTGTGTGGGATGTATTAAAAGGAGAGATTGTTGGAAAATTAGAAGGTCATGGTGGCAGAGTCACTGGTGTGCGCTCGAGT  
5 CCAGATGGGTTAGCTGTATGTACAGGTTTCATGGGACTCAACCATGAAAATATGGTCTCCAGGTTATCAATAG

## SEQ ID No. 14

Ste4-o17 Protein sequence (mutant)

10 MAHQMDSITYSNNVTQQYIQPQSLQDISAVEEEIQNKIEAARQESKQLHAQINKAKHKIQDASLFQMANKVTSLTKNKIN  
LKPNIIVLKGHNKISDFRWSRDSKRILSASQDGFMLIWDSASGLKQNAIPLDSQWVLSCAISPSTLVASAGLNNNCTIY  
RVSKENRVAQNVASIFKGHTCYISDIEFTDNAHILTASGDMTCALNDIPKAKRVREYSDHLGDLALAIPEEPNLENSN  
TFASCGSDGYTYIWDSRSPSAVQSFYVNDSDINALRFFKDGMSIVAGSDNGAINMYDLRSDCSIATFSLFRGYEERTPTP  
TYMAANMEYNTAQSPQTLKSTSSSYLDNQAVSLDFSASGRLMYSCYTDIGCVVWDVLKGEIVGKLEGHGGRVTGVRSSP  
15 DGLAVCTGSWDSTMKIWSPGYQ

SEQ ID No. 15 is presented in Figure 3A as Dbl Hu.

SEQ ID No. 16 is presented in Figure 3A as Cdc24 Sc.

SEQ ID No. 17 is presented in Figure 3A as Cdc24-m1.

20 SEQ ID No. 18 is presented in Figure 3A as Cdc24-m2.

SEQ ID No. 19 is presented in Figure 3A as Cdc24-m3.

MRC Laboratory of Molecular  
Biology,  
Hills Road,  
Cambridge.  
CB2 2QH

FOR THE PURPOSES OF PATENT PROCEDURE

PCT/GB 98 / 0 3 0 33

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT  
issued pursuant to Rule 7.1 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
identified at the bottom of this page

<b>I. IDENTIFICATION OF THE MICROORGANISM</b>	
Identification reference given by the DEPOSITOR:  Escherichia coli (CMK603) pRS414Cdc24(wt)	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:  NCIMB 40898
<b>II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION</b>	
The microorganism identified under I above was accompanied by:  <input type="checkbox"/> a scientific description  <input type="checkbox"/> a proposed taxonomic designation  (Mark with a cross where applicable)	
<b>III. RECEIPT AND ACCEPTANCE</b>	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 3 October 1997 (date of the original deposit) <sup>1</sup>	
<b>IV. RECEIPT OF REQUEST FOR CONVERSION</b>	
The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)	
<b>V. INTERNATIONAL DEPOSITARY AUTHORITY</b>	
Name: NCIMB Ltd 23 St Machar Drive Aberdeen Scotland Address: UK AB2 1RY	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 8 October 1997

<sup>1</sup> Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

MRC Laboratory of Molecular  
Biology,  
Hills Road,  
Cambridge.  
CB2 2QH

RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

CT/GB 98 / 0 3 0 3 3

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT  
issued pursuant to Rule 7.1 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
identified at the bottom of this page

<b>I. IDENTIFICATION OF THE MICROORGANISM</b>	
Identification reference given by the DEPOSITOR:  Escherichia coli (CMK603) pRS414cdc24-m1	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:  NCIMB 40899
<b>II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION</b>	
The microorganism identified under I above was accompanied by:  <input type="checkbox"/> a scientific description  <input checked="" type="checkbox"/> a proposed taxonomic designation  (Mark with a cross where applicable)	
<b>III. RECEIPT AND ACCEPTANCE</b>	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 3 October 1997 (date of the original deposit) <sup>1</sup>	
<b>IV. RECEIPT OF REQUEST FOR CONVERSION</b>	
The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)	
<b>V. INTERNATIONAL DEPOSITARY AUTHORITY</b>	
Name:  NCIMB Ltd 23 St Machar Drive Aberdeen Scotland UK AB2 1RY	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 6 October 1997

<sup>1</sup> Where Rule 6.4(d) applies, such date is the date on which the status of international depositary  
authority was acquired.

MRC Laboratory of Molecular  
Biology,  
Hills Road,  
Cambridge.  
CB2 2QH

RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

CT/GB 98 / 0 3 0 3 3

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT  
issued pursuant to Rule 7.1 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
identified at the bottom of this page

<b>I. IDENTIFICATION OF THE MICROORGANISM</b>	
Identification reference given by the DEPOSITOR:  Escherichia coli (CMK603) pRS414cdc24-m2	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:  NCIMB 40900
<b>II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION</b>	
The microorganism identified under I above was accompanied by:  <input type="checkbox"/> a scientific description  <input checked="" type="checkbox"/> a proposed taxonomic designation  (Mark with a cross where applicable)	
<b>III. RECEIPT AND ACCEPTANCE</b>	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 3 October 1997 (date of the original deposit) <sup>1</sup>	
<b>IV. RECEIPT OF REQUEST FOR CONVERSION</b>	
The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)	
<b>V. INTERNATIONAL DEPOSITARY AUTHORITY</b>	
Name: <b>NCIMB Ltd</b> <b>23 St Machar Drive</b> <b>Aberdeen</b> Scotland Address: <b>UK AB2 1RY</b>	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 6 October 1997

<sup>1</sup> Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

MRC Laboratory of Molecular  
Biology,  
Hills Road,  
Cambridge.  
CB2 2QH

RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE

PCT/GB 98 / 0 3 0 33

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT  
issued pursuant to Rule 7.1 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
identified at the bottom of this page

<b>I. IDENTIFICATION OF THE MICROORGANISM</b>	
Identification reference given by the DEPOSITOR:  Escherichia coli (CMK603) pRS414cdc24-m3	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:  NCIMB 40901
<b>II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION</b>	
The microorganism identified under I above was accompanied by:  <input type="checkbox"/> a scientific description  <input checked="" type="checkbox"/> a proposed taxonomic designation  (Mark with a cross where applicable)	
<b>III. RECEIPT AND ACCEPTANCE</b>	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 3 October 1997 (date of the original deposit) <sup>1</sup>	
<b>IV. RECEIPT OF REQUEST FOR CONVERSION</b>	
The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)	
<b>V. INTERNATIONAL DEPOSITARY AUTHORITY</b>	
Name:  NCIMB Ltd 23 St Machar Drive Aberdeen Scotland Address: UK AB2 1RY	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 6 October 1997

<sup>1</sup> Where Rule 6.4(d) applies, such date is the date on which the status of international depositary  
authority was acquired.

BUDAPEST TREATY ON THE INTERNATIONAL  
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE

MRC Laboratory of Molecular Biology,  
Hills Road,  
Cambridge.  
CB2 2QH

INTERNATIONAL FORM

VIABILITY STATEMENT  
issued pursuant to Rule 10.2 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
identified on the following page

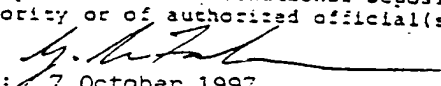
NAME AND ADDRESS OF THE PARTY  
TO WHOM THE VIABILITY STATEMENT  
IS ISSUED

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name:  Address: AS ABOVE	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:  NCIMB 40898 Date of the deposit or of the transfer:  3 October 1997
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on 6 October 1997 <sup>1</sup> . On that date, the said microorganism was <sup>2</sup> <input checked="checked" type="checkbox"/> <sup>3</sup> viable <input type="checkbox"/> <sup>3</sup> no longer viable	

- <sup>1</sup> Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
- <sup>2</sup> In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.
- <sup>3</sup> Mark with a cross the applicable box.

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED<sup>4</sup>

V. INTERNATIONAL DEPOSITARY AUTHORITY

<p>Name:</p> <p>Address: <b>NCIMB Ltd</b> 23 St Machar Drive Aberdeen Scotland UK AB2 1RY</p>	<p>Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):</p> <p></p> <p>Date: 7 October 1997</p>
---	--

<sup>4</sup> Fill in if the information has been requested and if the results of the test were negative.



BUDAPEST TREATY ON THE INTERNATIONAL  
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE

MRC Laboratory of Molecular Biology,  
Hills Road,  
Cambridge.  
CB2 2QH

INTERNATIONAL FORM

VIABILITY STATEMENT  
issued pursuant to Rule 10.2 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
identified on the following page

NAME AND ADDRESS OF THE PARTY  
TO WHOM THE VIABILITY STATEMENT  
IS ISSUED

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name:  Address: AS ABOVE	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCIMB 40899 Date of the deposit or of the transfer:  3 October 1997
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on 6 October 1997 <sup>1</sup> . On that date, the said microorganism was <sup>2</sup> <input checked="" type="checkbox"/> <sup>3</sup> viable <input type="checkbox"/> <sup>3</sup> no longer viable	

- <sup>1</sup> Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
- <sup>2</sup> In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.
- <sup>3</sup> Mark with a cross the applicable box.

CT/GB 98 / 0 3 0 3 3

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED<sup>4</sup>

V. INTERNATIONAL DEPOSITARY AUTHORITY

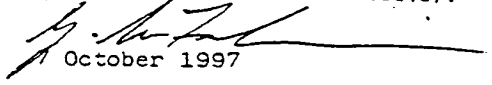
Name:

**NCIMB Ltd**

Address:

23 St Machar Drive  
Aberdeen Scotland  
UK AB2 1RY

Signature(s) of person(s) having the power  
to represent the International Depositary  
Authority or of authorized official(s):

Date:  October 1997

<sup>4</sup> Fill in if the information has been requested and if the results of the test were negative.

BUDAPEST TREATY ON THE INTERNATIONAL  
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE

MRC Laboratory of Molecular Biology, INTERNATIONAL FORM  
Hills Road,  
Cambridge.  
CB2 2QH

VIABILITY STATEMENT  
issued pursuant to Rule 10.2 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
identified on the following page

NAME AND ADDRESS OF THE PARTY  
TO WHOM THE VIABILITY STATEMENT  
IS ISSUED

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name:  Address: AS ABOVE	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCIMB 40900  Date of the deposit or of the transfer:  3 October 1997
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on 6 October 1997 <sup>1</sup> . On that date, the said microorganism was <div style="display: flex; justify-content: space-between;"> <div data-bbox="120 1262 293 1314"> <input checked="" type="checkbox"/> <sup>3</sup> viable         </div> <div data-bbox="120 1335 435 1388"> <input type="checkbox"/> <sup>3</sup> no longer viable         </div> </div>	

<sup>1</sup> Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

<sup>2</sup> In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.

<sup>3</sup> Mark with a cross the applicable box.

ST/GB 98 / 0 3 0 3 3

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED<sup>4</sup>

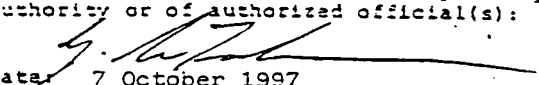
V. INTERNATIONAL DEPOSITARY AUTHORITY

Name:

Address:

NCIMS LTD  
23 St Machar Drive  
Aberdeen Scotland  
UK AB2 1RY

Signature(s) of person(s) having the power  
to represent the International Depositary  
Authority or of authorized official(s):

  
Date: 7 October 1997

<sup>4</sup> Fill in if the information has been requested and if the results of the test were negative.

BUDAPEST TREATY ON THE INTERNATIONAL  
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE

MRC Laboratory of Molecular Biology,  
Hills Road,  
Cambridge.  
CB2 2QH

INTERNATIONAL FORM

VIABILITY STATEMENT  
Issued pursuant to Rule 10.2 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
identified on the following page

NAME AND ADDRESS OF THE PARTY  
TO WHOM THE VIABILITY STATEMENT  
IS ISSUED

DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name:  Address: AS ABOVE	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCIMB 40901 Date of the deposit or of the transfer:  3 October 1997
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on 6 October 1997 <sup>1</sup> . On that date, the said microorganism was <input checked="" type="checkbox"/> <sup>3</sup> viable <input type="checkbox"/> <sup>3</sup> no longer viable	

- <sup>1</sup> Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
- <sup>2</sup> In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.
- <sup>3</sup> Mark with a cross the applicable box.

PL/GB 98 / 0 3 0 33

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED<sup>4</sup>

--

INTERNATIONAL DEPOSITARY AUTHORITY

Name:

Address:

**NCIMB Ltd**  
23 St Machar Drive  
Aberdeen Scotland  
UK AB2 1RY

Signature(s) of person(s) having the power  
to represent the International Depositary  
Authority or of authorized official(s):

Date: 7 October 1997

<sup>4</sup> Fill in if the information has been requested and if the results of the test were negative.

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/03033

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/31 C07K14/82 A61K38/16

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>ZHAO, Z.-S. ET AL.: "Pheromone signalling in <i>Sacharomyces cerevisiae</i> requires the small GTP-binding protein Cdp42p and its activator CDC24"</p> <p>MOLECULAR AND CELLULAR BIOLOGY, vol. 15, no. 10, October 1995, pages 5246-57, XP002089989</p> <p>cited in the application</p> <p>see abstract</p> <p>see page 5246, right-hand column, paragraph 2; figure 8</p> <p style="text-align: center;">--- -/-</p>	<p>1,2, 7-16, 19-26</p>

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

### \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

14 January 1999

Date of mailing of the international search report

01/02/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Smalt, R

# INTERNATIONAL SEARCH REPORT

Intern:      Application No  
PCT/GB 98/03033

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MIYAMOTO, S. ET AL.: "A Db1-homologous region of the yeast CLS4/CDC24 gene product is important for Ca <sup>2+</sup> -modulated bud assembly" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 181, no. 2, 1991, pages 604-10, XP002089990 see figure 1	1,3,13, 15,25
A	CERIONE, R.A. ET AL.: "The Db1 family of oncogenes" CURRENT OPINION IN CELL BIOLOGY, vol. 8, no. 2, April 1996, pages 216-22, XP002089991 cited in the application see figures 2,3; table 1	
P,X	NERN, A. ET AL.: "A GTP-exchange factor required for cell orientation" NATURE, vol. 391, 8 January 1998, pages 195-198, XP002089992 see the whole document	1-8, 11-14, 23-25, 27,28



# INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB 98/03033

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Claims 26 and 29, referring to an agent capable of affecting a GEF/G-beta interaction, could not be searched to completion due to insufficient characterization of the compound.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**THIS PAGE BLANK (USPTO)**



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/31, C07K 14/82, A61K 38/16</b>		<b>A1</b>	(11) International Publication Number: <b>WO 99/18213</b>
			(43) International Publication Date: 15 April 1999 (15.04.99)
(21) International Application Number: PCT/GB98/03033		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 8 October 1998 (08.10.98)		<b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> <i>With an indication in relation to deposited biological material furnished under Rule 13<sup>bis</sup> separately from the description.</i>	
(30) Priority Data:			
9721357.3      8 October 1997 (08.10.97)      GB 9721358.1      8 October 1997 (08.10.97)      GB 9812793.9      12 June 1998 (12.06.98)      GB			
(71) Applicant (for all designated States except US): MEDICAL RESEARCH COUNCIL [GB/GB]; 20 Park Crescent, London W1N 4AL (GB).			
(72) Inventors; and (75) Inventors/Applicants (for US only): ARKOWITZ, Robert, Alan [US/GB]; Division of Cell Biology, MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH (GB). NERN, Peter, Michael, Aljoscha [DE/GB]; Division of Cell Biology, MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH (GB).			
(74) Agents: HARDING, Charles, Thomas et al.; D. Young & Co., 21 New Fetter Lane, London EC4A 1DA (GB).			
(54) Title: MUTANTS OF YEAST Cdc24p, DEFECTIVE IN BINDING OF THE G-PROTEIN BETA SUBUNIT			
(57) Abstract			
<p>The pheromone signal transduction in yeasts involves hormone binding to a G-protein coupled membrane receptor, interaction of Cdc24p with G<math>\beta</math>, and ultimately results in polarized growth towards the hormone source (mating partner), caused by changes in the cytoskeleton. The present invention describes three recessive mutants of Cdc24p, at the amino acids 189 and 190, which do not interact with G<math>\beta</math>, and which cause the cytoskeleton to focus adjacent to the last budding site, rather than towards the hormone gradient. In contrast to previously described Cdc24p mutants, those presented here are not affected in their normal vegetative growth and hormone-induced processes, other than cytoskeleton orientation.</p>			
<pre> graph TD     Pheromone --&gt; Receptor[Pheromone receptor]     Receptor --&gt; Galpha[Gα]     Galpha --&gt; Gbetagamma[Gβγ]     Gbetagamma --&gt; Cdc24[Cdc24]     Cdc24 --&gt; Cdc42[Cdc42]     Cdc42 --&gt; Reorientation[Cytoskeletal Reorientation]     Reorientation --&gt; Growth[Cell Growth Towards Pheromone]           </pre>			

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		